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**HYPERCOAGULABILITY IN MULTIPLE  
MYELOMA AND MONOCLONAL  
GAMMOPATHY OF UNDETERMINED  
SIGNIFICANCE**

**BY  
THØGER NIELSEN**

DISSERTATION SUBMITTED 2020



**AALBORG UNIVERSITY**  
DENMARK



# **HYPERCOAGULABILITY IN MULTIPLE MYELOMA AND MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE**

by

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**AALBORG UNIVERSITY**  
DENMARK

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# ENGLISH SUMMARY

Cancer patients are associated with a greater risk of developing venous thromboembolism (VTE) than the general population. Multiple myeloma (MM) is a cancer that forms in the bone marrow and terminates in an increased VTE-risk. Its asymptomatic precursor, also known as monoclonal gammopathy of undetermined significance (MGUS), also have an increased VTE risk, although with slightly reduced intensity. The underlying mechanisms required to elucidate the amplified thrombosis formation lacks a conversant understanding, but may include increased levels of blood inflammatory and coagulation factors, hyperviscosity, acquired activated protein C resistance, and treatment. Recently, extracellular vesicles (EVs), small submicron particles with a lipid bilayer released from various cells, have been found to carry procoagulant phospholipids (PPL) and tissue factor (TF), the main initiator of the coagulation system. Investigation of EVs and their potential role in hemostasis is challenging due to their size and heterogeneity and they have proven difficult to isolate and analyze without interference from other blood components. Limited knowledge exists on the potential role of EVs in the disease-related VTE-risk of MM and MGUS. Therefore, this thesis focused on three studies, where hypercoagulability in MM and MGUS was evaluated, and a prospective model for investigation of procoagulant EVs were optimized and applied.

In study 1, we document hypercoagulability in platelet-free plasma from both MM and MGUS patients, where thrombin generation, PPL activity, and microvesicle-associated TF activity are increased. Some of the MM patients also exhibited increased levels of cell-free deoxyribonucleic acid indicative of neutrophil extracellular trap formation. Thrombin generation did not reveal any clear changes during and after anti-myeloma treatment. In study 2, we optimized a model for isolation of EV subpopulations from healthy subjects through differential ultracentrifugation and subsequent investigation of their procoagulant properties. We demonstrated that the isolated EVs were capable of increasing thrombin generation and PPL activity. In study 3, we identified increased levels of different EV subpopulations in MM patients. We found elevated TF and PPL activity related to EVs, possibly the larger microvesicles, in MM patients compared to healthy controls. After the first course of treatment, the procoagulant effect of the EVs diminish, especially in the patients eligible for high-dose chemotherapy with autologous stem cell support.

The results presented in this thesis contribute to the standardization of methods, together with its related analytical pitfalls, related to the investigation of procoagulant EVs. Our studies contributes to the understanding pertaining to the the complexity of the hypercoagulability associated with MM and MGUS, and, along with other scientific contributions, address the possible need for personalized thromboprophylaxis in MM. We highlight the potential role of procoagulant EVs in



this disease-related hypercoagulability and perhaps in the increased VTE-risk that MM patients hold.





# DANSK RESUME

Det er kendt, at patienter med kræft i noget hyppigere grad udvikler venøs tromboemboli (VTE) end den generelle befolkning. Myelomatose (MM), er en kræftsygdom i knoglemarven, som øger risikoen for at udvikle VTE markant. Monoklonal gammopati af ukendt signifikans (MGUS), et asymptomatisk forstadium til MM, har ligeledes en forøget risiko for udvikling af VTE, dog ikke i lige så høj grad. Nogle af de mulige mekanismer, der er med til at forårsage den øgede trombedannelse, inkluderer øgede mængder af blodets inflammations- og koagulationsproteiner, hyperviskositet, aktiveret protein C resistens og anti-myelomatosebehandling. I de seneste år har der været en stigende interesse i ekstracellulære vesikler (EV), som er små partikler, der udskilles af flere forskellige celletyper. EVs har en dobbelt lipidmembran og det har vist sig, at de er bærere af prokoagulante fosfolipider (PFL) og proteinet tissue factor (TF), som er den primære igangsætter af koagulationssystemet. Undersøgelsen af EV og deres potentielle rolle i hæmostase er udfordrende af natur pga. deres størrelse og heterogenitet. De er tilmed svære at isolere og analysere uden interferens fra andre af blodets komponenter. Meget lidt er beskrevet omkring EV og deres mulige rolle i den sygdoms-relaterede risiko for VTE i MM og MGUS. Dette ph.d.-projekt er baseret på tre studier, hvor vi undersøgte hyperkoagulabilitet i MM og MGUS samt optimerede og anvendte en model til undersøgelse af prokoagulante EV.

I studie 1 fandt vi tydelige tegn på hyperkoagulabilitet i trombocyt-frit plasma fra både MM og MGUS patienter. Dette omfatter øget trombingenerering, PFL-aktivitet og mikrovesikel-associeret TF-aktivitet. Nogle MM patienter havde også et øget niveau af celle-frit deoxyribonukleinsyre, hvilket kan antyde dannelse af neutrofile ekstracellulære fælder. Behandling af myelomatose syntes ikke have nogen tydelig indflydelse på trombingenereringen. I studie 2 optimerede vi en model til at isolere EV fra raske kontrolpersoner ved brug af differential ultracentrifugering med henblik på videre undersøgelse af deres prokoagulante egenskaber. De isolerede EV viste sig egnede til at fremme både trombingenerering og PFL-aktivitet. I studie 3 identificerede vi forskellige populationer af EV i MM patienter. De isolerede EVs, mestendels de større af slagsen, fra MM patienter viste sig at fremme TF- og PFL-aktivitet i modsætning til EV fra raske kontrolpersoner, der ingen effekt havde. Efter første behandling reduceredes den prokoagulante effekt af EV, specielt i den gruppe af MM patienter, der var egnede til højdosis kemoterapi med stamcellestøtte.

Resultaterne i denne ph.d.-afhandling bidrager til standardiseringen af metoder til undersøgelse af prokoagulante EV og de problemstillinger der følger med disse. Vores studier bidrager også til forståelsen af den kompleksitet den hyperkoagulabilitet, som MM og MGUS associeres med, og kan, sammen med andre videnskabelige bidrag, ydermere adressere det potentielle behov for personaliseret tromboseprofylakse i MM. Vi belyser samtidig de prokoagulante EV's rolle i den sygdomsrelaterede

hyperkoagulabilitet og muligvis i den øgede risiko for tromboser, som MM patienter har.

# ACKNOWLEDGEMENTS

My Ph.D. project rooted in the coagulation and thrombosis research, which for some time has been of great interest at the Department of Clinical Biochemistry at Aalborg University Hospital. Initially, I was involved in some pilot studies on M-proteins and their involvement in hypercoagulability in patients with multiple myeloma. However, I was soon drawn towards the exciting and, at the time, quite early research that was going on within the field extracellular vesicle (EV) in our research group. Coagulation was already one of the focus areas of the research group, and with the emerging interest in EVs, it was almost inevitable that a combination of the two would happen. This resulted in a Ph.D position surrounding EVs, thrombosis and multiple myeloma with my two supervisors, Shona Pedersen and Søren Risom Kristensen, and a collaboration with Henrik Gregersen and the Department of Hematology at Aalborg University Hospital. During my time as Ph.D. student at Aalborg University Hospital, I have been working amongst dedicated scientists and medical staff who have been forthcoming and truly helpful with almost any issues I have encountered.

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*Thøger Nielsen*

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## Reading guide

Three papers are listed on the following page, which is the product of the three studies that was intended for this Ph.D thesis. This page is followed by an *Abbreviations list* that summarizes all the abbreviations introduced in parantheses throughout the thesis.

The *chapters 1, 2 and 3* will cover the fundamental background and topics related to the three papers. *Chapter 1* provides for a description of the patient groups investigated in this thesis, namely multiple myeloma and its precursor monoclonal gammopathy of undetermined significance. *Chapter 2* describes hemostasis and the underlying mechanisms that may lead to thrombosis due to the comorbidity of both multiple myeloma and monoclonal gammopathy of undetermined significance. *Chapter 3* introduces extracellular vesicles and their emerging role as important ‘players’ in coagulation and potential contributors to thromboembolic complications in pathological conditions such as cancer.

The hypotheses and aims of the three studies of the thesis are introduced in *Chapter 4* and the methodological setup behind the studies are elaborated in *Chapter 5*.

The outcomes of each study are summarized in short in *Chapter 6* with referral to the papers attached in the *Appendix* section. *Chapters 7 and 8* unfolds in a discussion of the scientific and clinical outcomes of the thesis and ends in a conclusion.

At the end of *Chapter 9* with perspectives, there is a full list of *references*.

## List of papers

This Ph.D. thesis is based on four papers published in international peer-reviewed journals:

### *Paper I:*

**Prothrombotic abnormalities in patients with multiple myeloma and monoclonal gammopathy of undetermined significance.**

Nielsen T, Kristensen SR, Gregersen H, Teodorescu EM, Nielsen JE, Pedersen S.  
*Submitted to Thrombosis Research, Januar 2020.*

### *Paper II:*

**Investigation of procoagulant activity in extracellular vesicles isolated by differential ultracentrifugation.**

Nielsen T, Kristensen AF, Pedersen S, Christiansen G, Kristensen SR.  
*Journal of Extracellular Vesicles, 2018; 7(1): 1454777.*  
*doi:10.1080/20013078.2018.1454777*

### *Paper III:*

**Extracellular vesicle-associated procoagulant phospholipid and tissue factor activity in multiple myeloma.**

Nielsen T, Kristensen SR, Gregersen H, Teodorescu EM, Christiansen G, Pedersen S.  
*PLoS ONE, 2019; 14(1): e0210835. doi:10.1371/journal.pone.0210835*

## Abbreviations list

20K	20,000 × g
100K	100,000 × g
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ALAT	Alanine-aminotransferase
APTT	Activated partial thromboplastin time
ARF6	ADP-ribosylation factor 6
ASCT	Autologous stem cell transplantation
CAT	Calibrated automated thrombogram
CD	Cluster of differentiation
cf-DNA	Cell-free deoxyribonucleic acid
CR	Complete response
CRP	C-reactive protein
CTI	Corn trypsin inhibitor
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
DUC	Differential ultracentrifugation
EPCR	Endothelial protein C receptor
ESCRT	Endosomal sorting complexes required for transport
ETP	Endogenous thrombin potential
EVs	Extracellular vesicles
F	Factor
FC	Flowcytometry
FIXa	Activated factor IX
FVa	Activated factor V
FVIIa	Activated factor VII
FVIIIa	Activated factor VIII
FXa	Activated factor X
FXIIIa	Activated factor XIII
GP	Glycoprotein
GTPases	Guanosine triphosphate enzymes
HDCT	High-dose chemotherapy
HSP	Heat-shock protein
ILVs	Intraluminal vesicles
IMiDs	Immunomodulatory imide drugs
IMWG	International Myeloma Working Group
INR	International normalized ratio

ISEV	International Society for Extracellular Vesicles
ISS	International staging system
MGUS	Monoclonal gammopathy of undetermined significance
MHC-I	Major histocompatibility complex I
miRNA	MicroRNA
MM	Multiple myeloma
MPL	Melphalan, prednisone, and lenalidomide
MPT	Melphalan, prednisone, and thalidomide
MPV	Melphalan, prednisone, and bortezomib
mRNA	Messenger RNA
MVBs	Multivesicular bodies
MVs	Microvesicles
Mz-IIa	Intermediate meizothrombin
NETs	Neutrophil extracellular traps
NTA	Nanoparticle tracking analysis
PAI-1	Plasminogen activator inhibitor-1
PAR-1	Protease-activated receptor-1
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PD	Progressive disease
PFP	Platelet-free plasma
PPL	Procoagulant phospholipids
PR	Partial response
PS	Phosphatidylserine
RhoA	Ras homolog family member A
RNA	Ribonucleic acid
sCR	Stringent complete response
SD	Stable disease
SEC	Size exclusion chromatography
SPP	Standard pooled plasma
TAFI	Thrombin activatable fibrinolysis inhibitor
TEM	Transmission electron microscopy
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TG	Thrombin generation
TGA	Thrombin generation assay
TM	Thrombomodulin
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
tPA	Tissue plasminogen activator
TSG101	Tumor susceptibility gene 101

VCD	Bortezomib, cyclophosphamide, and dexamethasone
VGPR	Very good partial response
VTE	Venous thromboembolism
vWF	von Willebrand factor
WB	Western blotting



# Chapter 1. Multiple myeloma

## Plasma cell disorders

The term plasma cell disorders refers to a group of disorders characterized by disproportionate proliferative growth of one clonal plasma cell<sup>1</sup>. A product of the clones is abnormal monoclonal proteins secreted in excess. The abnormal proteins are the second common denominator for the plasma cell disorders, and are often, referred to as paraproteins or M-proteins. The M-proteins are detectable in the blood and urine as either monoclonal intact immunoglobulins, immunoglobulin fragments and/or free light and/or heavy chains. The severity of gammopathies vary from completely benign to malignant conditions, which can be asymptomatic or (more or less) symptomatic diseases<sup>1</sup>. The benign version of the disorders is the asymptomatic and mostly non-progressive condition called monoclonal gammopathy of undetermined significance (MGUS). Malignant plasma cell disorders are categorized as either asymptomatic or symptomatic, but both are progressive stages. Smouldering myeloma is asymptomatic, but the plasma cell clones have evolved into an aggressive subtype. If the clones further evolve, the disease progresses to the stage of symptomatic and more aggressive version called multiple myeloma (MM) requiring treatment. Ultimately, and only in rare occasions, the multiple myeloma may evolve into the most malignant stage of plasma cell leukemia<sup>2</sup>.

## Epidemiology

MM is a relatively uncommon form of cancer and for normal individuals, the average lifetime risk of developing MM is below 1%. However, in some parts of the world, ethnicity is a major contributor to increased risk of MM<sup>3</sup>. It is the second most frequent cancer of the hematological cancers, where it comprises approximately 10-20% of all new cases<sup>4,5</sup>. In 2016, it was estimated that approximately 25,000-30,000 new cases of MM will be detected annually<sup>6-8</sup>. In a worldwide perspective, MM is accountable for roughly 1% of all new cases of cancer per year and is the cause of 1% of all cancer-related deaths each year<sup>9</sup>. Age is a risk factor for MM and therefore it is a disease of the elderly, where the representative median age is between 65 to 70 years<sup>6,10</sup>. It seldom occurs in people less than 30 years of age<sup>3,11</sup>. The prevalence of MM is expected to escalate over time, since the life expectancy of the general population will increase<sup>12</sup>. Other known risk factors are family history of multiple myeloma or a personal history of MGUS. Immunodeficiency and autoimmune diseases have also been suggested as other risk factors, but the underlying data is contradictory and the coherence is left unresolved<sup>13</sup>. In 99% of the cases, MM is preceded by the premalignant MGUS disorder<sup>14</sup>. MGUS is a rather common condition and accounts for more than 3% of the general white population with an age of 50 years or more<sup>15</sup>. Although patients with MGUS do not require treatment, they may be at continuous

risk of progression. Each year, 1% progress, usually to one of several malignant conditions that require treatment, among these are MM<sup>16,17</sup>.

## **Pathophysiology/Pathogenesis**

MGUS resembles MM by having clonal plasma cells infiltrating the bone marrow and secrete M-proteins into the blood, however, to a much lesser extent<sup>1</sup>. The multistep process leading MGUS to MM is complex and even though the etiology on some points are well-documented, it is poorly understood on others. The transition into MM requires several oncogenic events to occur in both the plasma cells and in the bone marrow microenvironment. These events include cytogenetic alterations that promote e.g. immunoevasion, drug resistance, increased osteolytic activity, dysregulation of the cell cycle, and apoptosis<sup>18–22</sup>. The stage of MM is characterized as a clonal B-cell neoplasm, where terminally differentiated monoclonal B-cells, i.e. the clonal plasma cells, undergo clonal expansion and invades the bone marrow at multiple sites (hence the name). Here, they continue proliferating and secreting increased levels of M-proteins<sup>23–26</sup>.

## **Clinical features and diagnosis**

The diagnosis of MGUS and MM is based on three criteria: concentration of M-protein in the blood, the amount of clonal plasma in the bone marrow, and the extent of organ damage. Bone pain is the one of the most common features of MM affecting more than 70% of the patients. The pain arises from osteolytic lesions and increased bone breakdown propagated by the cancerous plasma cells that secretes factors that activate the osteoclasts to break down the bone faster than normal, thus skewing the bone remodeling balance<sup>27,28</sup>. In the aftermath of increased bone breakdown, hypercalcemia arises. Hypercalcemia is a common complication in MM at diagnosis, but this frequency has recently decreased probably due to a faster and earlier diagnostic approach<sup>10,29</sup>. Hypercalcemia can lead to renal insufficiency, which occurs in approximately one out of four patients. It has been documented that free light chain proteinuria can also contribute to this<sup>30</sup>. Another common clinical feature of MM is anemia that is evident in 40-70% of MM cases<sup>10,31</sup>. The anemia can in part be attributed to the invasion of plasma cells in the bone marrow, but also be due to changes in the cytokine environment, resulting in impaired erythropoiesis<sup>32</sup>. MM patients are at increased risk getting infections, mainly due to hypogammaglobulinemia and renal insufficiency<sup>33,34</sup>. It has been reported that infection is responsible for approximately half of the MM-related deaths<sup>35</sup>. Other clinical features may include amyloidosis and protein loss, especially albumin.

Suspicion of MM should be aroused when a patient present with one or more of the above mentioned clinical features. Particularly hypercalcemia, renal insufficiency, anemia, and bone lesions (CRAB-features) are important in the distinction between MGUS, smouldering myeloma, and MM. The International Myeloma Working Group

(IMWG) released in 2003 (with updates in 2014) a subset of recommended diagnostic criteria for MM and these are now used in the diagnosis MGUS, smouldering myeloma, and MM<sup>36,37</sup>. The IMWG criteria are listed in Table 1.

**Table 1. Diagnostic criteria for diagnosis of MGUS, smouldering myeloma, and multiple myeloma.**

MGUS	Smouldering myeloma	Multiple myeloma
M-protein (type IgG or IgA) in serum < 30 g/L <b>or</b> M-protein in urine < 500 mg/day <b>and</b> < 10% clonal plasma cells in the bone-marrow <b>and</b> No symptoms or no CRAB criteria met <b>and</b> No basis for other B-cell disease, primary AL-amyloidose, or light chain/heavy chain or immunoglobulin-associated tissue damage	M-protein (type IgG or IgA) in serum > 30 g/L <b>and/or</b> M-protein in urine ≥ 500 mg/day <b>and/or</b> ≥ 10% clonal plasma cells in the bone-marrow <b>and</b> No symptoms or no CRAB criteria met	M-protein in serum and/or urine regardless of concentration* <b>and</b> ≥ 10% clonal plasma cells in the bone-marrow or plasmacytoma in histological biopsy from cancerous tissue <b>and</b> One or more CRAB-criteria met

\*In case of non-secretory myeloma, M-protein in the blood or urine are undetectable, however, free light chains are detectable in irregular ratios.

## Classification

The prognostic assessment of MM is tricky by nature, since the disease is heterogeneous. A significant reason for this the underlying chromosomal anomaly and can be ascribed to cytogenic alterations that can identify patients at high risk<sup>38,39</sup>. Moreover, in 1975, Durie and Salmon introduced a staging system for MM that was designed to define the tumor burden and survival based on common clinical features<sup>40</sup>. This system became obsolete after the introduction of the International Staging System (ISS) with improved reproducibility, where the quantitative levels of  $\beta_2$ -microglobulin play an important role<sup>41,42</sup>, see Table 2. Previously, the 5-year survival rate in multiple myeloma was around 20% with a range of survival spanning from a couple of years to more than 10 years. However, since the beginning of the century, the survival rate has increased in parallel to the increasing effectiveness of treatment<sup>6</sup>.

**Table 2. The International Staging System for multiple myeloma**

Stage I	Stage II	Stage III
Serum $\beta_2$ -microglobulin < 3.5 mg/L and albumin $\geq$ 3.5 g/dL	Neither stage I or III, meaning either: Serum $\beta_2$ -microglobulin 3.5 to < 5.5 mg/L irrespective of the serum albumin levels  <b>or</b> Serum $\beta_2$ -microglobulin < 3.5 mg/L but serum albumin < 3.5 g/dL	Serum $\beta_2$ -microglobulin $\geq$ 5.5 mg/L
Median survival is 62 months*	Median survival is 44 months*	Median survival is 29 months*

\*These data are adapted from Greipp et al<sup>41</sup>.

## Treatment

Today, MM patients receive different treatment regimens based on their age, general health situation, eligibility to future stem cell transplantations, and cancer aggressiveness (genetic type). Autologous stem cell transplantation (ASCT) has a risk of toxic (even fatal) complications, thus many patients are not candidates for such a treatment due to comorbidities. Several countries use an age limit as a deciding factor in the decision process to offer a patient ASCT and high-dose chemotherapy (HDCT) or not<sup>43</sup>. Furthermore, several protocols for treatment of MM, using various combinations of drugs, exists across the world. Lately, the consensus regarding treatment regimens are becoming more consistent.

The common anti-myeloma treatment regimen for patients eligible for ASCT include induction therapy, HDCT with stem cell support, consolidating treatment (if needed), and maintenance therapy (if needed). The general consensus is to prime with a three-drug induction therapy beforehand, rather than two-drug regimens<sup>44,45</sup>. Bortezomib is typically one of the drugs used, as it increases the overall survival<sup>46</sup>. Generally, an early HDCT (typically using high-dose melphalan) with ASCT is recommended to improve treatment response (see Table 3/appendix) and postpone disease progression<sup>47,48</sup>. The primary treatment of MM for patients ineligible for ASCT consists of several multidrug combinations. Usually, the treatment is a combination of corticosteroids (dexamethasone or prednisone) and non-chemotherapy drugs, like

immunomodulatory imide drugs (iMiDs), and proteasome inhibitors. The combinations vary from melphalan and prednisone in combination with thalidomide, lenalidomide, or bortezomib (MPT, MPL, and MPV, respectively) to lenalidomide/revlamide in combination with dexamethasone. Lenalidomide in combination with dexamethasone is associated with an increased overall survival and progression free survival compared to the other regimens<sup>49</sup>. However, when using lenalidomide or thalidomide, thromboprophylactic treatment is recommended, due to the increased risk of developing thrombosis<sup>50</sup>. In contrast, other studies report of higher overall survival for the MPV regimen compared to the one using lenalidomide and dexamethasone<sup>51</sup>. This may be a result of the addition of bortezomib to the MPV regimen, which has shown to improve overall survival and postpone disease progression, although at the cost of increased neurotoxicity<sup>52</sup>. Novel proteasome inhibitors and iMiDs have been investigated and it has been reported that these agents increase the tolerability and clinical outcome. Furthermore, newer targeted therapies, like the monoclonal antibody daratumumab, are showing great potential in regards of treatment response and remission status and have a lower toxicity level<sup>53</sup>.

**Table 3. Multiple myeloma response criteria (modified from IMWG)**

<b>Stringent complete response (sCR)</b>	CR as defined below
	Normal FLC ratio
	Absence of clonal plasma cells in bone marrow by immunohistochemistry or 2- to 4-color flow cytometry
<b>Complete response (CR)</b>	No M-protein detectable in serum or urine by electrophoresis or immunofixation
	< 5% plasma cells in bone-marrow No soft tissue plasmacytomas
<b>Very good partial response (VGPR)</b>	M-protein detectable in serum or urine by electrophoresis or immunofixation ≥ 90% reduction in serum M-protein and urine M-protein < 100 mg/day
<b>Partial response (PR)</b>	50% reduction of serum M-protein and reduction in daily urinary M-protein by ≥ 90% or to < 200 mg/day
	If serum and urine M-protein are not measurable, a ≥ 50% decrease in the difference between involved

	<p>and uninvolved FLC levels is required in place of the M-protein criteria</p> <p>If serum and urine M-protein are not measurable, and free light assay is also not measurable, <math>\geq 50\%</math> reduction in bone-marrow plasma cells is required in place of M-protein, provided baseline percentage was <math>\geq 30\%</math></p> <p>In addition to the above criteria, if present at baseline, <math>\geq 50\%</math> reduction in the size of soft tissue plasmacytomas is also required</p>
<b>Stable disease (SD)</b>	Not meeting criteria for CR, VGPR, PR, or PD
<b>Progressive disease (PD)</b>	<p>Increase of 25% from lowest response value in any of the following:  ~ Serum M-protein (absolute increase must be <math>\geq 0.5</math> g/dL), and/or  ~ Urine M-protein (absolute increase must be <math>\geq 200</math> mg/day), and/or  ~ Only patients without measurable serum and urine M-protein levels: the difference between involved and uninvolved FLC levels (absolute increase must be <math>\geq 10</math> mg/dL)  ~ Only in patients without measurable serum and urine M-protein levels and without measurable disease by FLC levels, bone marrow plasma cell percentage (absolute percentage must be <math>\geq 10\%</math>)</p> <p>Definite development of new bone lesions or soft tissue plasmacytomas or definite increase in the size of existing bone lesions or soft tissue plasmacytomas</p> <p>Development of hypercalcemia (corrected serum calcium <math>&gt; 11.5</math> mg/dL or <math>2.65</math> mmol/L) that can be attributed solely to the plasma cell proliferative disorder</p>

## Consequences of multiple myeloma

While the anti-myeloma treatments improve, but the prevalence of MM rises, it is important to take care of other complications that accompanies MM resulting in

serious consequences and increased mortality. Many patients experience symptoms like fatigue, bone pain, dehydration and increased frequency of infections. However, the more serious complications vary from neurologic disorders (e.g. like dementia), pathologic fractures, organ failure, bleeding abnormalities, to severe infections among others<sup>54</sup>. A devastating complication of MM is venous thromboembolism (VTE) that additionally contributes to the increased morbidity and mortality in these patients. It is moreover evident that the risk of VTE is prevalent in patients with MGUS<sup>55,56</sup>. In this PhD project, the mechanisms and associative role of VTE in patients with MM and MGUS will be addressed in detail in the next section of the thesis.

## Chapter 2. Venous thromboembolism

### Introduction

Centuries ago, both Richard Wiseman and Rudolf Virchow separately contributed to the fundamental understanding of venous thrombosis and pulmonary embolism<sup>57</sup>. Their work culminated in the advent of three major factors potentially contributing to the risk of thrombosis. The triad, entitled Virchow's triad, describes three vital risk factors, namely hypercoagulability, endothelial dysfunction (vessel wall injury), and hemodynamic changes (blood stasis/turbulence)<sup>57,58</sup>. Today, VTE is the world's one of the most frequent cardiovascular disorders, resulting in a significant reduction in quality of life to eventual termination in death<sup>59,60</sup>. Hospitalization is believed to be responsible for more than 50% of the cases of VTE, allegedly due to surgery, inflammation, and immobilization that are directly associated with two of three factors in Virchow's triad, namely vessel wall injury and blood stasis<sup>61</sup>. Hypercoagulability, the third factor, is associated with underlying diseases, e.g. inflammatory diseases and cancer<sup>62</sup>.

### The hemostatic system

The role of the hemostatic system is to repair and keep the vessels intact in the cardiovascular system. Under certain circumstances, like VTE events, the vital blood flow to the different organ systems are impaired and a subset of complex hemostatic processes are set in motion to enact bleeding arrest and repair in case of vascular injury<sup>63</sup>. In this section, the general processes of blood clotting will be introduced, followed by a more detailed description of several key mechanisms that regulate the hemostatic system under normal and pathological situations.

### The process of blood clotting

The process of blood clotting is divided into primary, secondary, and tertiary hemostasis and in general, it covers platelet aggregation, coagulation, and fibrinolysis<sup>64</sup>. The primary hemostasis is the phase, where damaged endothelial cells secrete von Willebrand factor (vWF) that binds to exposed collagen, platelets are recruited and they adhere to vWF at the site of injury and becomes activated due to stimulus through several endothelial and subendothelial adhesion proteins. The activated platelets begin to adhere to each other and the primary platelet plug is formed<sup>65,66</sup>. In secondary hemostasis, the end product is cross-linked fibrin that acts as stabilizer for the primary platelet plug in case of more severe vessel damage. The formation of fibrin is highly dependent on the initiation of the blood coagulation system. Coagulation is comprised of two different pathways, namely the intrinsic, extrinsic, that share a common pathway, where several coagulation factors (F) interact



through a series of reactions<sup>67</sup>. The primary initiator of secondary hemostasis is tissue factor (TF) in the extrinsic pathway that will initiate a sequence of tenase reactions leading to an initial burst of thrombin generation (TG)<sup>68</sup>. Through several positive feedback mechanisms involving the intrinsic, common pathway, and activated platelets, TG will rapidly increase, ultimately resulting in the conversion of fibrinogen to fibrin. Fibrin will polymerize and form a fibrin clot that strengthens the platelet clot formed during primary hemostasis<sup>63</sup>. In a normal person, minor injuries are common and hemostasis is a constant ongoing process, but the process is also constantly halted or reverted by of coagulation inhibitors. However, if the clot formation were to remain after successful vessel repair, the vasculature bed would be constantly obstructed with blood clots. Therefore, fibrinolysis occurs to break down the fibrin clots and this phase is known as tertiary hemostasis. Tertiary hemostasis covers the dissolution of the fibrin clot through protease activity exerted by plasmin<sup>69</sup>. At the beginning of any vessel injury, the fibrinolysis is inhibited by thrombin, but once the damage is reduced or repaired, TG declines and the fibrinolytic processes are initiated. In short, plasminogen is converted to plasmin at the site of injury and thus the fibrin clot is dissolved. The hemostatic processes are highly complex and if a situation of vascular injury or arrest arises, many factors are involved to restore normal flow of blood and repair of subendothelial and endothelial tissue.

### **Endothelial cells and leukocytes**

The endothelium that line the tunica intima of all blood vessels serve as the first ‘gate keeper’ between the blood and the surrounding tissue. It consists of endothelial cells that help regulate the extravasation and blood fluidity and smooth muscle cells that facilitate vasoconstriction and -dilation<sup>70</sup>. In case of inflammation, endothelial cells help recruit leukocytes to the site and increase the leukocyte infiltration<sup>71</sup>. Under normal conditions, they serve as anti-coagulants through platelet inhibition with prostacyclin and nitric oxide<sup>72</sup>. The endothelial cells further inhibit coagulation activity by secreting tissue factor pathway inhibitor (TFPI) and the three endothelial receptors thrombomodulin (TM), endothelial protein C receptor (EPCR), and protease-activated receptor 1 (PAR-1). PAR-1 that are involved in activated protein C cleavage of activated FV (FVa) and activated FVIII (FVIIIa)<sup>72,73</sup>. In case of vascular injury endothelial cells exert important procoagulant features. If stimulated by vasoactive agents, e.g. histamine or thrombin, shear fluid stress or inflammatory cytokines, the endothelial cell release so-called Weibel-Palade bodies to the blood. These organelles mainly contain vWF that binds to exposed collagen and are important for platelet adhesion to the endothelium, but also P-selectin and E-selectin involved in platelet rolling prior to adhesion<sup>72,74</sup>.

### **Platelets**

When vessel injury occurs, the platelets are exposed to subendothelial matrix proteins that makes them adhere to the site of injury, either directly or indirectly. The direct

adhesion to the endothelium happens via the cell surface receptors like glycoprotein (GP) VI and integrin  $\alpha 2\beta 1$ , which both binds to collagen<sup>75</sup>. Indirect platelet adhesion is mediated by the GP1b-IX-V receptor complex on platelets binding to the collagen-bound vWF secreted by the endothelial cells<sup>76,77</sup>. The binding of platelets to the subendothelial matrix proteins also activates the platelets. The activation of platelets induces change of shape, degranulation, membrane flipping, release of procoagulant extracellular vesicles (EVs), and increases cell signaling<sup>78</sup>. In this phase, the platelets increase in size by becoming elongated with cytoplasmic extensions due to cytoskeletal reorganization of the actin filaments<sup>79</sup>. Simultaneously, the anionic phospholipid, phosphatidylserine (PS), is flipped, from mainly being exposed on the inner membrane leaflet, to the outer through flippase activity<sup>80</sup>. Owing to its negative charge, PS is the platform on which the coagulation cascade occurs and thus, is essential for the secondary hemostasis<sup>81</sup>. The platelets also contain cytoplasmic granules that they release upon activation. The granules,  $\alpha$ -granules being the most abundant, contain various proteins involved in hemostasis and inflammatory processes, e.g. fibrinogen, FV, FVIII, vWF, and P-selectin<sup>82</sup>. The platelets also secrete dense granules that contain adenosine diphosphate (ADP), thromboxane  $A_2$ , and serotonin, capable of activating other thrombocytes<sup>83</sup>. Furthermore, the intracellular calcium levels increase resulting in increased cell signaling, which too is important for recruitment of additional platelets, but also for the binding of fibrinogen to the GPIIb/IIIa receptor that allows platelets to aggregate to one another<sup>84</sup>. Platelet recruitment and aggregation create the platelet plug, which in most cases is sufficient to stop bleeding in small vessels, but if the trauma is more severe, secondary hemostasis involving tenase activity, complex formation, and fibrin generation, is required.

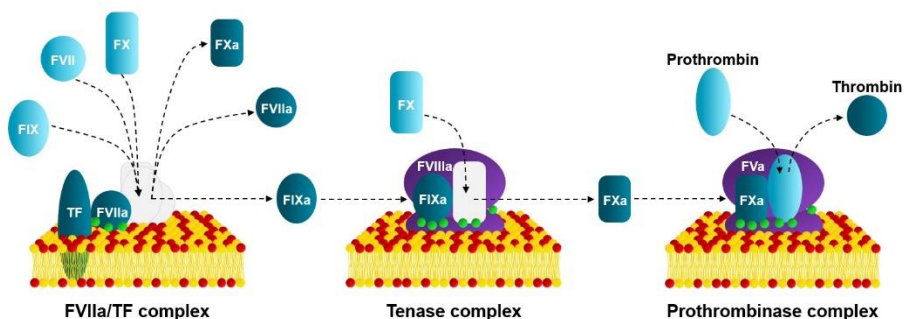
### **Tissue factor**

In the coagulation cascade, TF is considered the key initiator of coagulation. TF exerts its role in the extrinsic pathway, also called the TF pathway, which takes place in the secondary hemostasis<sup>85</sup>. TF, also known as FIII, is a GP consisting of 263/261 amino acids that contains three domains: the extracellular domain involved in complex formation, the transmembrane domain that serves as anchor in the membrane, and a cytoplasmic domain that participate in signal transduction<sup>86,87</sup>. High levels of TF are present in astroglial cells and epithelial cells surrounding organs, and the vascular adventitia surrounding blood vessels larger than capillaries<sup>88</sup>. Other cell types, such as smooth muscle cells and endothelial cells, only express noteworthy amounts of TF once stimulated by inflammatory proteins<sup>89-91</sup>. Upon vessel damage, subendothelial TF is exposed to the blood by the perivascular fibroblasts. Here, it will form a complex with circulating FVIIa and together they form the TF-FVIIa complex<sup>68,92</sup>. The TF-FVIIa complex activates FX to the active form (FXa) through tenase reactions, and FXa in turn activates the co-factor, FVa, and forms the prothrombinase complex. The prothrombinase complex converts the initial burst of prothrombin to thrombin, which ultimately will amplify its own production<sup>93</sup>. Blood-borne TF is mainly found in

monocytes and macrophages upon appropriate stimulation. Studies have proclaimed that neutrophils and eosinophils also may contribute to this, however, the levels of TF in these cell types may be very low and possibly insignificant<sup>94</sup>. Another main source of blood-borne TF is TF embedded in the membrane of circulating microvesicles (MVs). TF associated to microvesicles (MVs) will be described in further detail in the chapter on EVs. A truncated soluble version of TF exists too; however, in this form TF exerts no coagulation activity<sup>95</sup>.

### Procoagulant phospholipids

The main source of procoagulant phospholipids (PPL) is activated platelets, and most abundant in a subpopulation of activated platelets, called balloon-shaped platelets<sup>96</sup>. A secondary source of PPL is the circulating MVs that are rich in external PS<sup>97</sup>. The predominant types of PPL are anionic PS and neutral phosphatidylcholine (PC). PPL-protein interactions on membrane surfaces are essential for blood clotting and may restrict the secondary hemostasis to the site of injury and/or inflammation<sup>98,99</sup>. The binding of coagulation proteins to PPL-rich surfaces may also promote anti-coagulation reactions by inhibiting thrombin formation, e.g. through protein C activity<sup>100</sup>. PPL allows for binding of several coagulation factors, and is essential for the formation of several complexes that in turn lead the excess TG. The previously described formation of the TF-FVIIa complex is assembled on the anionic PPL surfaces when  $\text{Ca}^{2+}$  is present<sup>101</sup>. In turn, the TF-FVIIa complex will convert FVII and other proenzymes, like FIX and FX, to their active forms. The active FIX (FIXa) creates the tenase complex with FVIIIa on the anionic PPL surface, and is a much more potent activator of FX, than the TF-FVIIa complex<sup>102</sup>. FXa will form another complex on the anionic surfaces, namely the prothrombinase complex, with FVa and finally convert prothrombin to thrombin in excess. Figure 1 illustrates the tenase reactions leading to thrombin generation and the role of anionic PPL surfaces in the process.



**Figure 1. The complex formation of coagulation factors (F) and the role of phosphatidylserine (PS) leading to thrombin formation.** The formation of the FVIIa/TF complex activates FIX, FX, and additional FVII. Activated FIX form the tenase complex with activated FVIII (which is activated by activated FX)

and together they are a potent activator of FX. FXa forms the prothrombinase complex with FVa and starts converting prothrombin to thrombin. The green dots represents PS. TF: Tissue factor.

### Thrombin generation and fibrin formation

This initial burst of TG is take place on the surface of fibroblasts that is being exposed to the blood due to vascular injury. The TF-FVIIa complex generates small initial burst of thrombin through intermediate meizothrombin (Mz-IIa), however, this only serves to activate platelets and initiate the coagulation cascade<sup>103</sup>. Once the amplification phase is initiated, the coagulation moves from fibroblasts to platelets for futher amplification of FXa. In this phase, the initial burst of thrombin and Mz-IIa generated activate FXI that in turn will activate FIX<sup>104</sup>. Simultaneously, thrombin and FXa activate the FVIII, the cofactor essential for the tenase complex formation and conversion of excess FXa<sup>104,105</sup>. Thrombin is also responsible for the activation of FV, the other vital constituent of prothrombinase complex<sup>106</sup>. In the next phase, the propagation phase, the prothrombinase complex creates a spike in TG, often referred to as an ‘explosive’ burst in TG, where more than 90% of the thrombin is generated<sup>107</sup>. More platelets will be activated by the thrombin and release coagulation factors from their intracellular stores of granules. The abundance of thrombin generated in the propagation phase will cleave fibrinogen to fibrin. Thrombin facilitates the cleavage of fibrinogen into fibrin monomers and FXIII, activated by thrombin, facilitates the polymerization of the fibrin monomers into fibrin polymer networks through cross-linking via isopeptide bonds<sup>108</sup>. To prevent the fibrin clot from being broken down, thrombin is also activating thrombin-activatable fibrinolytic inhibitor (TAFI)<sup>109</sup>. Finally, a fibrin clot is created, which help stabilize the platelet plug formed during primary hemostasis. Conversely, thrombin may also bind to TM on endothelial cells and peripheral blood cells and in turn activates protein C. Protein C then inactivates the tenase and prothrombinase complexes, by cleaving FVIIIa and FVa on the anionic surfaces<sup>110,111</sup>.

### Fibrinolysis

The main ‘player’ in fibrinolysis is the enzyme plasmin that is responsible for breakdown of the fibrin clot. In response to injury, the damaged endothelial cells secrete tissue plasminogen activator (tPA) along with other plasminogen activators to the blood. These cleave fibrin-bound plasminogen to plasmin that is co-located to fibrin<sup>112</sup>. In this way, the plasmin specifically lyses and degrades the cross-linked fibrin clot alone and not other proteins in the fluidic phase. Several degradation products are released to the blood during this process, one of them being D-dimer, a laboratory marker for fibrinolysis after recent coagulation activity<sup>113</sup>. Fibrinolysis is regulated by a subset of inhibitors, including the aforementioned TAFI in the clot developmental phase<sup>109</sup>. The two most dominant inhibitors of fibrinolysis is  $\alpha$ 2-antiplasmin and plasminogen activator inhibitor-1 (PAI-1) originating from platelet granules and the liver<sup>112,114</sup>.

## Emerging factors with procoagulant interest

While the hemostatic processes are divided into three well-defined segments, many other factors are believed to influence specific parts of the system. NETosis is a process, where nuclear material is released by neutrophil cells to the extracellular space. The meshwork consists mainly of DNA coiled around histones, but do also include proteins from cytoplasmic granules and together these are referred to as neutrophil extracellular traps (NETs)<sup>115</sup>. The purpose of NETs formation is believed to be associated to antimicrobial defence mechanisms where they catch and eliminate pathogens<sup>116</sup>. NET are either formed through a process known as late suicidal NETosis that results in death for the neutrophil, or through early vital NETosis, where the neutrophil stay alive and exert other immune-related functions like chemotaxis and phagocytosis. During late suicidal NETosis, the neutrophils are exposed to prolonged stimuli, e.g. pathogens, interleukins or antibodies, production of reactive oxygen species and a downstream cascade of enzymatic conversions, involving PAD-4, lead to decondensation of the nucleus and histone citrullination<sup>117</sup>. Simultaneously, neutrophil elastase (NE) and myeloperoxidase are released from intracellular granules and enters the nucleus and starts unfolding the chromatin and eventually result in disruption of the nucleic membrane<sup>118</sup>. The chromatin is now decorated with cytosolic and granular proteins and are released to the extracellular space through a process believed to involve disruption of the cellular membrane<sup>118,119</sup>. Early vital NETosis, on the other hand, is a fast occurring process (within minutes) and is closely related to infectious stimuli, like bacteria or complement proteins<sup>120</sup>. The unfolding of chromatin and disruption of the nucleic membrane occurs in a similar manner as in late suicidal NETosis<sup>120,121</sup>. DNA-strands, however, are released from the nucleus through nuclear-envelope blebbing, and thus, the nucleus stays intact<sup>121</sup>. The DNA-containing vesicles are then released at the cellular membranes and NET formation occurs in the extracellular space<sup>121</sup>. Recently, NETosis has also shown to play a role in noninfectious diseases where it may contribute to cancer metastasis, inappropriate coagulation, and thrombosis<sup>122,123</sup>. For instance, histones H3 and H4 activate and aggregate platelets and activated platelets release thromboxane A2 that triggers NETosis. As a result of this cycle of events, the overall TG increases in a platelet-dependent manner<sup>124,125</sup>. NETs may also bind FXII, which then autoactivates through contact activation, and initiates fibrin formation via the intrinsic pathway<sup>126</sup>. Furthermore, neutrophil serine proteases, like NE and cathepsin G, has shown to inactivate TFPI by cleaving it and consequently promote the procoagulant activity<sup>127</sup>. NETs may also prevent fibrin degradation through inhibition of tPA<sup>128</sup>. NETs, as the name indicates, can trap other procoagulant factors that circulates in the blood. For instance is NETs proposed to trap procoagulant EVs that carry TF and PS<sup>129,130</sup>. EVs and their origin, biogenesis, release mechanisms, and role in the coagulation system will be further elucidated in the next chapter. The chapter will be concluded with some insight on how to isolate and study EVs, particularly in relation to procoagulant extracellular vesicles.

## Venous thromboembolism in multiple myeloma

Almost two centuries ago, French physicians Jean-Baptiste Bouillaud and Armand Trousseau manifested the first causal association between cancer and thrombosis<sup>131</sup>. Today, it is well-known that cancer is associated with increased risk of VTE and that the absolute risk is closely connected to stages of cancer, anti-cancer treatment, and type of cancer<sup>132,133</sup>. The VTE-risk in cancer patients are reported as 4-5 times higher than those without. Some cancers entail an even higher risk of developing VTE, e.g. lung and gastrointestinal tract cancers<sup>133,134</sup>. Patients with hematological cancers probably have one of the highest VTE-risks, which are reported as high as 28-fold increased<sup>134</sup>. Among the hematological cancers, MM entails a high VTE-risk, as does the precursor, MGUS<sup>55,135–137</sup>. The risk of developing VTE for patients with MM and MGUS is highest within the first year after diagnosis<sup>56</sup>. A large retrospective study from 2010 showed, that patients with MM displayed a 7.5-fold increased risk of VTE after a one-year follow-up and 4.1-fold increased risk after a 10-year follow-up period<sup>138</sup>. The pathogenesis for the increased MM- and MGUS-specific VTE-risk mostly remains unclear. Multiple factors, including interactions between the comorbidities, tumor-related factors, and the treatment regimen, have been proposed as contributors to the heightened VTE-risk in patients with MM<sup>50,139</sup>. General risk factors for VTE, such as age, surgery, immobilization, underlying chronic conditions, inherited thrombophilia and cancer in general, are also of relevance in patients with MM<sup>135,140</sup>. Biochemical risk factors, such as increased vWF, increased FVIII, acquired activated protein C resistance, decreased protein S levels, have also been proposed as possible candidates capable of inducing hypercoagulability<sup>141,142</sup>. It has further been proposed that the excessive production of the M-component may be associated with the procoagulant state in MM<sup>141,143,144</sup>. So far, no study has detected any valid biomarkers for the VTE-risk in MM and MGUS. Cancer treatments in general is a known discrete risk factor of VTE<sup>145</sup>. Anti-myeloma is no exception, especially in the early stages of the therapy. Furthermore, therapies that includes the IMiDs, like thalidomide and lenalidomide, heightens the risk of thrombotic complications during treatment of MM and are therefore typically accompanied by thromboprophylaxis<sup>146–148</sup>. A few studies have indicated that EVs, carrying procoagulant factors like TF and PPL, are potential candidates for biomarkers in MM-associated VTE-risk and hypercoagulability<sup>149–151</sup>.

## Chapter 3. Extracellular vesicles

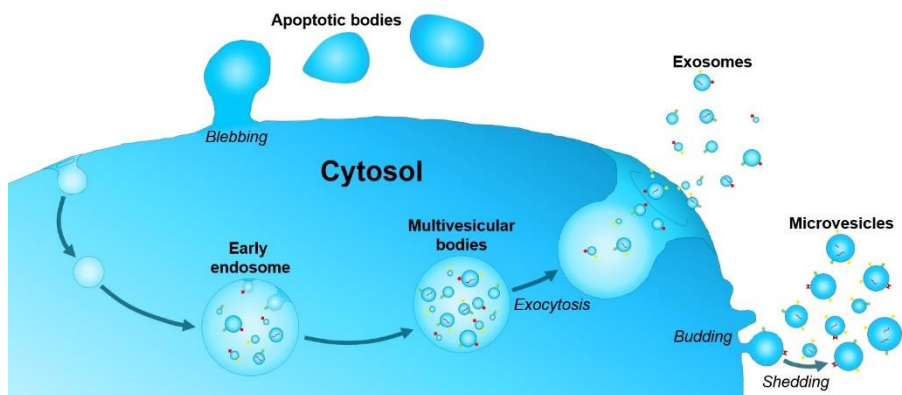
### Discovery and state of the art

The transfer of information from one cell to another is an important feature for the growth and maintenance. Intercellular communication is typically mediated through direct cell-to-cell contact or through secretion of signaling molecules for the recipient cell to interact with. In recent decades, another option for cells to communicate emerged, and this involves the intercellular transfer of EVs. Erwin Chargaff and Randolph West described the first signs of EVs in 1946, as pellets, produced through ultracentrifugation, exerting procoagulant activity<sup>152</sup>. Almost 20 years later, Peter Wolf, identified vesicular structures through ultracentrifugation and electron microscopy, and refers to these as ‘platelet dust’, and shortly after, Harrison Clarke Anderson identifies matrix vesicles in regards to calcification processes<sup>153,154</sup>. It has long been known that cells undergoing apoptosis, as part of the tissue homeostasis, release apoptotic bodies that are large vesicular structures with a diameter typically between 1-5  $\mu\text{m}$ . More recently, the intercellular communication seems to be mostly associated with EVs released from healthy cells, which comprise a diameter in the range of roughly 40-200 nm. Throughout the years many different types of EVs has been identified and their origin varies from many different cell types and body fluids, like blood, urine, breast milk, seminal fluid, saliva among others<sup>155–159</sup>. In 2011, the International Society for Extracellular Vesicles (ISEV) was established to accumulate data, optimize analyses, increase clinical impact, and unify the nomenclature on the topic of EVs.

### Nomenclature, biogenesis, and molecular composition

Although being tiny fragments of their host organism, EVs are complex and highly heterogeneous entities in terms of size, protein composition, and origin. This undoubtedly do complicate the nomenclature, which are highly reflected in the literature. In recent years scientists (and ISEV) have reached some consensus regarding the nomenclature. The collective name for all vesicles released by the host cells are termed EVs, however, two main EV-subtypes were defined, namely exosomes and MVs. These terms were created in light of the origin, genesis, and mechanism of release of the EVs, as depicted in Figure 2. Exosomes, that generally constitute the smallest of EVs, are assembled in multivesicular bodies (MVBs) that fuse with the cell membrane and release its content to the extracellular space. They are believed to have a diameter of 40-100 nm and a density ranging from 1.13 to 1.19 g/mL, but they may also present with a larger diameter that overlaps with that of MVs. MVs are believed to present with a diameter > 100-200 nm and a density of 1.25-1.30 g/mL and is shed from the originating cell through direct budding of the plasma membrane<sup>160,161</sup>. Their size may be as large as 1-2  $\mu\text{m}$ <sup>162</sup>. Similar to the exosomes, the

estimated diameter is not a universal rule, because smaller vesicles of 100 nm in diameter may also bud from the cell membrane. Because of overlap in size, it is difficult to clearly distinguish between MVs and exosomes on size alone. In order to identify specific exosomes or MVs, one must also consider an in-depth analysis of e.g. surface proteins or cargo that may point towards a specific biogenesis and release mechanism.



**Figure 2. Release paths of exosomes, microvesicles, and apoptotic bodies.** Inward budding of the plasma membranes create early endosomes. Further inward budding of the membrane of the early endosomes form multivesicular bodies, which upon fusion with the plasma membrane releases their content of exosomes. Microvesicles is directly formed by an initial outward budding of the plasma membrane and are ultimately shed to the plasma. Apoptotic bodies are formed by outward blebbing of the plasma membrane as result of the apoptotic disassembly of dying cells.

The release of EVs from cells require certain different stimuli dependent on cell of origin. Platelets release EVs upon activation and this may be triggered through direct binding to thrombin or collagen<sup>163,164</sup>. Endothelial cells activated by cytokine stimulation, e.g. tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), or through interaction with reactive oxygen species may also secrete EVs<sup>165,166</sup>. Molecules released by bacteria, e.g. liposaccharide, stimulate dendritic cells to release EVs<sup>167</sup>. B, T, and natural killer cells can release EVs upon activation of cell surface receptors<sup>168</sup>. A fundamental trigger mechanism for the release of EVs, however, seem to be the accumulation or ‘spike’ of intracellular calcium levels<sup>169,170</sup>. Other known stimulants include factors like hypoxia, complement stimulation, irradiation, changes in pH, and cellular stress<sup>171,172</sup>. Cancer cells also secrete EVs upon stimulation, e.g. as a response to anti-cancer treatment, to aid the cancer cells in exerting certain malignant features, like metastasis, angiogenesis, and avoiding immunodetection among others<sup>173,174</sup>.



## Exosomes

Exosomes are formed within the endosomal network, where the process starts with endosome formation through inward budding of the MVB. Late endosomes undergo invagination of their membranes resulting in multiple intraluminal vesicles (ILVs)<sup>175</sup>. The process of ILV-formation highly depends on the endosomal sorting complex required for transport (ESCRT) complex<sup>176,177</sup>. The ESCRT complex is a multifaceted protein machinery that is specialized in promotion of the membrane budding process, cargo sorting, and formation of the MVBs. The complex constitutes four separate proteins, ESCRTs 0 through III, and is involved in sorting and incorporation of receptors and other endosomal membrane proteins as well as engulfment of cytosolic proteins into the ILVs<sup>176,177</sup>. Lipids and tetraspanins facilitate other ESCRT-independent sorting pathways that participate in the receptor sorting and cargo selection process. The tetraspanins, e.g. CD81, are found in omnipresent tetraspanin-enriched-microdomains and helps in the compartmentalization of several receptors and membrane-associated signaling molecules<sup>178,179</sup>. The lipid-dependent pathway depends on formation of lipid rafts that serves as conversion site for sphingomyelin to ceramide through sphingomyelinases. The ceramide-enriched domains causes inward bending of the endosomal membranes<sup>180,181</sup>.

The constitutive elements of exosomes are largely dependent on the cell of origin. According to ExoCarta and EVpedia, they include more than 4000 proteins, 200 lipids, 2000 mRNAs and miRNAs<sup>182,183</sup>. However, some proteins are more prominent than others and several of these are often used as markers to identify exosome populations. The tetraspanins CD9, CD63, CD81, and CD82, which are involved in cell penetration, adhesion, and fusion, are examples of such proteins<sup>184</sup>. Other markers used to identify exosome populations include proteins involved in exosomal release, e.g. tumor susceptibility gene 101 (TSG101) and Alix, or heat shock proteins (HSP) involved in antigen-related activity, e.g. HSP70<sup>184</sup>. Exosomes released by many cells have a high content of cholesterol, sphingomyelin, and ceramides, but a low content of PC.

## Microparticles

MVs are not formed in the endosomal network in the cytoplasm of cells, but is generated through shedding of the cell membrane<sup>185</sup>. Much is still unknown about the mechanisms behind the biogenesis of MVs, but several underlying mechanisms have been proposed. MV formation is facilitated by ‘budding’ of the plasma membrane and requires several diverse and localized changes in the membrane structure to occur. This includes reorganization of lipid and protein composition that alters the membrane curvature and rigidity and finally involves the actin-myosin compartment of the cytoskeletal network<sup>186</sup>. The plasma membrane is under normal circumstances constituted by an extracellular and a cytoplasmic leaflet, i.e. the outer and inner leaflet, respectively. The two leaflets are very different in their electrostatic potential due to

their lipid composition. PC and sphingomyelin are enriched on the outer leaflet, whereas PS, phosphatidylinositol, and phosphoinositides predominately are found in the inner leaflet<sup>187,188</sup>. An important step in the formation and release of MVs is breaking the asymmetry between the outer and inner leaflet. This process is facilitated by translocase activity, where adenosine triphosphate (ATP)-dependent flippases and floppases and ATP-independent scramblases are the most important categories of proteins<sup>80,189</sup>. The flippases mainly translocate phospholipids from the outer to the inner leaflet, the floppases translocate in the reverse direction, and scramblases act as bidirectional translocators through random phospholipid distribution<sup>80,187</sup>. The final fission and release from the cell membrane rely on kinase-mediated (e.g. calpain) cleaving of the cytoskeletal compartments and an ATP-dependent contraction facilitated by the actin-myosin machinery<sup>180</sup>. ADP-ribosylation factor 6 (ARF6), as well as several Rho guanosine triphosphate enzymes (GTPases). Particularly Ras homolog family member A (RhoA), plays an important role in phosphorylating a kinase system at the neck of the budding vesicle that leads to activation of actin-myosin machinery<sup>180,190</sup>.

The sorting of protein and nucleic acid cargo in MVs is a complex and selective process that is driven through oligomerization and recruitment of proteins in the cytoplasm. ARF6 is one of the proteins that drives selective recruitment of cargo proteins. It is important in the process of incorporating integrins and major histocompatibility complex I (MHC-I) into the MVs<sup>191</sup>. The lipid composition of MVs are rather unique, especially due to the PS on the outside of the vesicle membrane, but other lipids like sphingolipids, acylcarnitines, and fatty acid esters are enriched in MVs<sup>192</sup>. Some suggestions to proteins present on medium and large EVs include some actinins, mitofilin and HSPs, like GP90<sup>193</sup>. Nevertheless, the current understanding of the protein and lipid composition of MVs, and how it relates to the plasma membrane of the originating cell, is vaguely described in contrast to the exosomes.

## Biological properties and functions

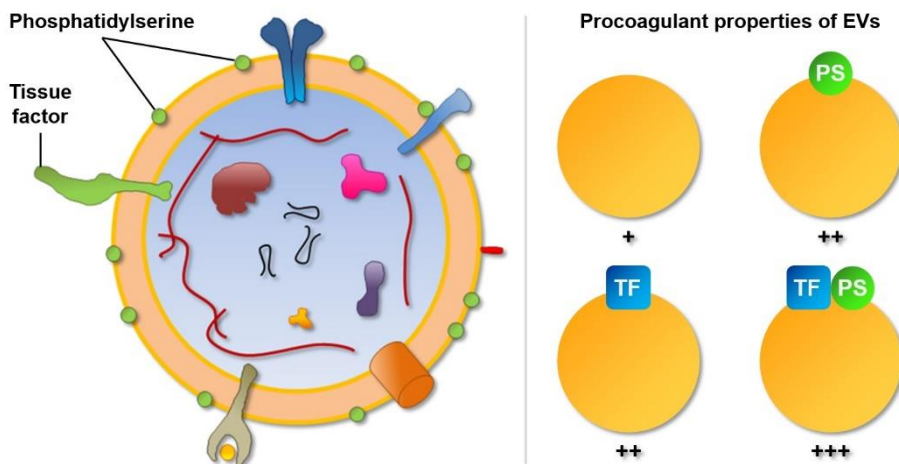
EVs are a way for cells to communicate with another through transfer of signaling molecules that alter functionality the recipient cell. Some of the functions EVs exert include neuron-glia communication, stem cell tissue repair, and immune modulation<sup>194–197</sup>. The way EVs act as messengers may be exerted through several mechanisms. They can release their cargo directly to the extracellular space, stimulate through direct cell surface receptor binding, fuse with the recipient cell, or undergo internalization through endocytosis<sup>185,198,199</sup>. Cell-to-cell communication via EVs is highly dependent on the protein composition on the surface of the EVs. Specific surface receptors and ligands are responsible for the binding to intended binding site, be it either specific recipient cells or the extracellular matrix. Upon binding to their designated targets, they may induce intracellular communication through various pathways, but the binding may also prompt for internalization of the EVs<sup>199</sup>. In order to release nucleic acids or proteins of cytoplasmic origin into the recipient cells, the

EVs must release their content by means of membrane fusion or endocytosis<sup>200</sup>. The bilipid layer of EVs is what makes them suitable as ‘shuttles’ for transport of functional and informative molecules. This is especially important for transport of informative nucleic acids, like mRNA and miRNA, that otherwise would be quickly degraded in the blood before reaching their destination<sup>201</sup>. The knowledge of the mechanisms behind where EVs are designated deliver information and how this occurs is still lacking and warrents caution when interpreting the communicative function of EVs<sup>180,200</sup>. In addition to cell communication, another important function of EVs is to eliminate unwanted molecules from the cells, such as amyloid proteins and modified RNA<sup>202,203</sup>. This role is further reflected in the rapid clearance of EVs from the circulation. The estimated half-life of EVs in blood are reported to be approximately 2 minutes, whereafter they are cleared from the body through the liver, spleen, and lungs<sup>204</sup>. EVs that are released from infected cells or cells affected by stressfull conditions, e.g. hypothermia, heat shock, hypoxia and oxidative stress, are thought to be involved in the trafficking of abnormal proteins and RNA content, including various viruses and prions<sup>205–207</sup>. In the past decades, EVs have been detected in many diseases and in several of these, specific EV populations, associated to the underlying disease, have been identified, e.g. cardiovascular diseases, metabolic diseases, infectious diseases, and neurological disorders<sup>208–211</sup>. EVs possess several pro-cancer abilities, like aiding in tumor progression and increased proliferation. EVs that contain growth factors and cytokines have shown to support angiogenesis, whereas EVs containing TF may aid the cancer cells in proliferation, immuno evasion and apoptosis, and support invasion, and angiogenesis<sup>212,213</sup>. However, another consequence of TF-bearing EVs is their procoagulant potency and a conceivable link to thrombosis.

### **Procoagulant extracellular vesicles**

Knowledge of the role of EVs in coagulation and development of thrombosis has lately increased markedly. Especially the MVs are of interest as significant ‘players’ in the hemostatic system, due to their biogenesis involving externalization of anionic phospholipids to the outer leaflet of the membrane. Of the externalized phospholipids, it is mostly PS that exert procoagulant activity by facilitating tenase complex binding, see Figure 3. Most MVs are PS-exposing, especially those secreted from activated platelets, but MVs that are PS-negative have been detected<sup>214,215</sup>. Other MVs carry TF in the membrane and these mainly released by monocytes and to some extent neutrophils. Endothelial cells, leukocytes and platelets has been reported to express TF, but there is some controversy on this topic<sup>94,97,216</sup>. The MVs carrying only PS are believed to have limited procoagulant activity (see Figure 3) and since they typically are released by activated platelets, they also carry receptors for vWF and collagen<sup>97</sup>. Therefore, they are also referred to as ‘mini-platelets’, and may have an intended purpose in hemostasis. The MVs that carry TF promote coagulation through the extrinsic pathway and are typically released after vessel injury to trigger blood coagulation and maybe clot growth as an alternate source of TF to aid in triggering

coagulation after injury<sup>97,216</sup>. Monocyte-derived MVs that contain both PS and TF are believed to exert highest level of procoagulant activity. They may be contributors to not only hemostasis, but also thrombosis and especially under pathological circumstances<sup>97</sup>. It has been suggested that MVs may exert procoagulant properties by other means than the extrinsic pathway. This include involvement of the intrinsic coagulation cascade in a FXI- and FXII-dependent manner<sup>217–219</sup>. However, the main understanding of EVs and their role in hemostasis and VTE can be generally attributed to TF and PS. EVs have also been described as having anti-coagulation abilities, because they can carry inhibitory proteins like TFPI, protein C, and protein S. This shed light on the complexity of the role that EVs have in terms of coagulation and further points out the delicate balance that the hemostatic system offers. Many types of cancer cells express high levels of TF on their surface and that many cancer patients have increased levels of circulating TF<sup>220,221</sup>. If this is a directly connected (the cancer cells secreting TF via MVs) or indirect (systemic response to the cancer) is obviously interesting topic in many types of cancer. Not much research has been made on procoagulant EVs in MM. Auwerda et al.<sup>149</sup> demonstrated procoagulant MV in MM patients, although without linking the MVs directly to the cancer cells. TF-production in clonal plasma cells have been described<sup>222</sup>, so it is an apparent to ask if there may be a connection between MM cancer cells and procoagulant EVs.



**Figure 3. The procoagulant properties of extracellular vesicles (EVs).** EVs carry various different cargo, e.g. lipids, and nucleic materials, that are encapsulated by a double-lipid membrane. Transmembrane tissue factor (TF) and phosphatidylserine (PS) embedded on the outer leaflet of the membrane is the two major contributors to EV-mediated procoagulant activity, especially in microvesicles. EVs positive for TF or PS have a role in hemostasis and may enhance coagulation, whereas EVs positive for both TF and PS may play a more pronounced role in both hemostasis and thrombosis.

## Investigation of extracellular vesicles

The investigation of EVs offers complexibility and comes along with a subset of preanalytical and analytical challenges and important considerations to make. EVs are secreted by many cells in the human body and therefore reside in several different human fluids, like urine, cerebrospinal fluid etc. Nevertheless, a very common source of EVs is the blood and typically is drawn from the antecubital vein using venipuncture, tourniquet, vacuum blood collection tubes, and a small diameter needle. Different types of processed blood samples exist, but serum and plasma is the most commonly studied blood source for EVs depending on downstream choice of analyses. Serum, for instance, is believed to contain a great proportion of EVs secreted by activated platelets in the clotting process occurring during the sampling process, which should be considered in respect to some functional analyses. Some issues regarding blood sample collection and handling involve the use of a tourniquet, the size of the needle, and vacuum tubes among others, since this may lead to endothelial damage and hemolysis. The temperature, agitation, and duration at which the blood samples are exposed to prior to plasma extraction may also affect the EV quantity and integrity. It is further recommended to snap-freeze samples for long-time storage of samples at maximum  $-80^{\circ}\text{C}$  and avoid frequent freeze-thaw cycles. It is therefore crucial that external factors like these are taken into consideration if they are not to interfere with and obliterate the original purpose of an investigation.

Since most human sample types are heterogeneous mixtures of cells, EVs, proteins, lipoproteins etc., most analyses of EVs require further isolation in order to effectively determine the composition of different EV-subpopulations and their physiological properties. This can be achieved through several isolation techniques, which all have advantages and disadvantages in regards to the downstream analysis plan.

### Isolation

Most research on EVs is using conditioned cell culture media, but the commonly used source for high-yield EV-isolation from body fluids is plasma, namely the type of plasma called platelet-free plasma (PFP)<sup>223</sup>. PFP is achieved through a double centrifugation at  $2,500 \times g$  for 15 minutes<sup>224</sup>. Purification of EVs from PFP and similar sample types can be time-consuming and usually include some loss of EVs as well as co-isolation of contaminants, like lipoproteins, protein aggregates and remnants of dead cells. Furthermore, some isolation techniques prompt for aggregation of EV and proteins, which will further complicate characterization and functional analysis of the EVs. The most frequently used methods for isolation of EVs from any fluid includes size exclusion chromatography (SEC), density gradient centrifugation, and filtration, but the most commonly used is ultracentrifugation and differential ultracentrifugation (DUC)<sup>223</sup>. Less commonly used isolation techniques include affinity, fluorescence liquid chromatographic separation. Some study limitations like sample volume and complexity, makes other isolation techniques, e.g. magnetic bead separation, to be

preferred over others. Because many of these techniques have some advantages and limitations in specific applications, many researchers use a combination of two or more isolation techniques. DUC may cause EVs to aggregate with one another or contaminants like lipoproteins and protein aggregates and it fail to isolate distinct size-dependent EV populations. Density gradient centrifugation may remove almost all protein contaminants, but co-isolates lipoproteins and remnants of similar density, not to mention that it is a time-consuming procedure<sup>223</sup>. SEC is a quick isolation technique that in contrast to the centrifugation techniques do not induce aggregation and damage of EVs and proteins. It may, however, often result in co-isolation of other vesicular structures, protein aggregates, and remnants of similar density and size<sup>223</sup>.

### Characterization

The process of quantitation and characterization of EVs opens a door to numerous different optical and non-optical techniques, many of which cannot stand alone in concluding quantity or specific EV subpopulations. A comprehensive survey was published by Gardiner et al.<sup>223</sup> describing the different common and less commonly tools used for characterization of EVs. Some methods are more often used than others and these include western blotting (WB), single-particle tracking methods, electron microscopy, and flow cytometry (FC), but like the isolation techniques they all come with specific advantages and limitations in relation to their application. Less commonly used techniques include protein assays, atomic force microscopy, enzyme linked immunosorbent assays, and procoagulant assays. In the category of electron microscopy techniques, transmission electron microscopy (TEM) is often used to detect and verify the presence of vesicular shapes encapsuled in a membrane according to the principle of ‘seeing is believing’. This method is sensitive to contaminants like lipoproteins, protein aggregates, and apoptotic bodies and immunogold labeling with specific antibodies against EV-markers or functional molecules often accompany TEM<sup>225</sup>. The most common method for single-particle tracking used is the nanoparticle tracking analysis (NTA) that covers both quantitation and size determination of particles in the sample with a diameter range of approximately 10-200 nm. Unfortunately, NTA do not only measure EVs, but also lipoproteins and protein aggregates, and can therefore not stand alone when used to analyze EV sizes and concentrations<sup>226,227</sup>. FC is often used in the quantitative and qualitative of EVs, but struggles to detect the smaller of EVs<sup>228</sup>. FC also allows for fluorescence detection of antibodies against specific EV-surface markers, thus describing various types of EVs. The conventional WB method is commonly used to detect specific proteins associated to the general markers for exosomes or MVs or specific subpopulations of EVs related to origin, function and/or disease<sup>223</sup>. When using WB though, it may be difficult to distinguish whether bands are detected because of co-isolation of proteins or not. Many use protein assays, like the bicinchoninic acid assay, to support other analyses, but this is also sensitive to protein contaminants. Some antibody micro arrays, like the EV Array<sup>229,230</sup>, are used to identify specific EV-populations based on surface markers, like tetraspanins, PS or

TF. In relation to PS and TF, some techniques exist to identify PS- and TF-positive EVs and their potential procoagulant effect.

### **Functional investigation of procoagulant extracellular vesicles**

Procoagulant EVs have been described in various diseases, including sepsis, diabetes, and not least cancer<sup>231–233</sup>. Most of the techniques used to study procoagulant activity of EVs resolves around coagulation assays used for investigation of plasma coagulation and many of these methods are not optimized for functional investigation of EVs and lack nuances. The calibrated automated thrombogram (CAT) is a global coagulation test that utilize plasma samples to depict TG. Even though a specialized trigger kit has been developed for CAT for the purpose of investigating MV-mediated TF activity, the likeliness of other influential factors in the plasma affecting the results is highly plausible. The PPL assays too, uses plasma to identify MV-associated PPL activity, but may likely also measure PPL from other sources like residual thrombocyte fragments and apoptotic bodies or PS trapped in NETs. However, both the CAT and PPL assays is believed to have potential in detecting EV-mediated procoagulant activity if they were combined with some kind of EV purification. Some studies have investigated similar concepts and ideas and showing its promising results<sup>231,234–237</sup>. Regardless, the concept still warrants further investigation and validation in order to achieve any evident clinical application. Other more EV-oriented coagulation assays have emerged throughout the years. Wang et al. described an stand-alone FXa-dependent assay capable of measuring MV-associated TF in EVs isolated by ultracentrifugation of plasma<sup>238</sup>. Connor et al. described a method to detect MV-associated PPL activity in EVs pelleted from through ultracentrifugation<sup>239</sup>. In general, the investigation of procoagulant EVs require a meticulous combination of more than one method for both identification of procoagulant markers and functional analysis of EV-mediated procoagulant activity.

## Chapter 4. Thesis Objectives

The underlying mechanisms that lead to hypercoagulability in cancer is complex and commonly multifactorial. Typically, a myriad of factors is out of balance and interacts with each other and other components of the hemostatic system. Eventually, it can lead to the development of thrombotic events, like deep vein thrombosis or pulmonary embolism. Recently, EVs have been identified as one such factor with procoagulant potential that may contribute to the increased VTE risk in cancer, especially in hematological cancers, like multiple myeloma. This gave rise to the following questions:

- Cancer patients, including MM, are in general at higher risk of developing VTE, but is it possible to detect individuals at high risk and administer thromboprophylactic treatment?
- Why do MM patients develop VTE and is it possible to detect hypercoagulability in these patients?
- If so, is this the hypercoagulability affected by procoagulant EVs and in what manner?

Therefore, the overall objective of the thesis has been to demonstrate a procoagulant state in patients with MM and MGUS for the purpose being able to administer thromboprophylaxis. We hypothesise that:

**Patients with MM and MGUS have increased procoagulant activity in the blood compared to healthy subjects.**

and

**Elevated blood levels of procoagulant EVs exist in patients with MM in comparison to healthy subjects.**

To investigate this, three studies with separate aims have been designed:

**Study 1:** To investigate the extent of hypercoagulability in plasma from patients with MGUS and MM at diagnosis and during two diverse anti-myeloma treatment regimens for the MM patients.

**Study 2:** To establish a model for isolation of EVs in plasma using DUC including a following assessment and validation of the model using quantitative and qualitative analyses. Furthermore, the procoagulant properties of the isolated EVs were investigated using several functional coagulation assays.



**Study 3:** To investigate the procoagulant activity of EVs in patients with MM using the model from study 2.

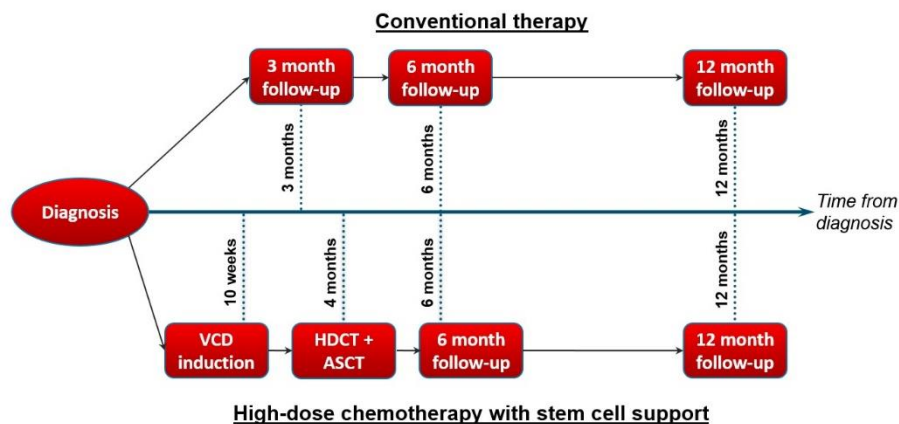
## Chapter 5. Materials and methods

### Study population

In this thesis, a total of 38 patients with MM and 19 patients with MGUS were recruited to the project at Department of Hematology at Aalborg University Hospital. The patients was diagnosed according to the guidelines published by the IMWG<sup>240</sup>. None of the enrolled patients had a history of VTE or other malignancy and received no anti-coagulation drugs at the time of the enrollment. At enrollment, the patients had their relative plasma cell levels in the bone marrow counted, lytic skeletal lesions assessed, and were staged according to the ISS<sup>41</sup>. The MM patients had a mean age of 71 years with a range of 40-87 and an approximate gender distribution of 50%. The MGUS patients had a mean age of 72 years ranging from 41-85) and had an approximate 50% gender distribution.

The first blood MM samples were drawn before initiation of treatment with biological drugs. Figure 4 illustrates the blood sampling procedure for the two treatment regimens the MM patients were assigned to. Patients younger than 70 years of age and in general good health were considered eligible for HDCT with ASCT in which case they were treated with a VCD induction therapy followed by HDCT with ASCT. Patients older than 70 years of age were regarded ineligible for ASCT and therefore received conventional therapy, mainly MPV. The treatment response were recorded in relation to the IMWG myeloma response criteria<sup>241</sup> everytime a sample was collected.

As control group, 34 healthy volunteers provided blood samples. The age and gender of the control subjects had a similar age and gender distribution as the patient groups, presenting with a gender distribution of approximately 50% of each gender, but with a mean age slightly lower (63 years, range 56-67) than the patient groups.



**Figure 4.** An overview of the blood sampling procedure of patients with multiple myeloma. After having submitted a blood sample at diagnosis, the patients submit blood samples at different time points dependent on their treatment regimen. Patients receiving conventional therapy (mainly melphalan-prednisone-bortezomib) submitted blood samples at 3-, 6-, and 12-month follow-ups. Patients eligible for high-dose chemotherapy (HDCT) and autologous stem cell transplantation (ASCT) submitted samples after an induction therapy consisting of bortezomib, cyclophosphamide, and dexamethasone (VCD) and after their HDCT+ASCT treatment. Thereafter, they submitted blood samples at their 6- and 12-month follow-up.

## Study 1

All 38 MM patients, 19 MGUS patients and 34 healthy control subjects were included in this study. Two MM patients were excluded due to following rediagnosis to smouldering myeloma. Five patients did not finish their treatment sampling schedule; four died from sepsis and one from unknown reasons. Two additional patients left the study due to severe illness and loss to follow-up.

## Study 2

Twelve healthy volunteers provided blood samples for this methodological study.

## Study 3

Twenty of the MM patients were included in this study. These had a mean age of 72 years (range 40-84) and 55% were males. The twenty control subjects included for this study presented with a mean age of 64 years (range 56-67) and 55% were males.

## Ethical considerations

The local scientific ethics committee of the North Denmark Region approved the protocol, approval N-20130075. Blood samples from patients MM patients, MGUS

patients, and healthy controls as described by research protocol linked to the approval number. Since the blood samples from patients with MM were collected in relation to the ongoing diagnosis and treatment, the same intravenous access (antecubital fossa) was used, which means that no additional needle prick necessary. The recruitment of patients with MGUS and controls, on the contrary, required the individual persons to attend to a blood collection, solely with purposes related to this study. Due to the small volume of blood collected, enrolment in the study was not considered to be of any significant risk for the patients and control subjects.

## **Sample collection and preparation**

All blood samples was collected by routine venipuncture from the median cubital vein at the outpatient clinic at the Department of Clinical Biochemistry, Aalborg University Hospital, through a BD Vacutainer Blood Collection Set (Becton Dickinson, Franklin Lakes, NJ, USA) with a 21-gauge needle, butterfly and tourniquet. The first couple of millilitres of blood was collected in a separate tube, which was discarded because of the activating effect of the tourniquet and potential fibroblast contamination<sup>242</sup>. The blood was collected in 6 ml 0.105 M (3.2%) buffered trisodium citrate BD Vacutainer (Becton Dickinson) and PFP was extracted through doublecentrifugation at  $2,500 \times g$  for 15 minutes at room temperature carefully leaving 1 cm above the buffy coat as suggested by Lacroix et al.<sup>224</sup>. The PFP was then aliquoted for the different analyses and frozen at  $-80^{\circ}\text{C}$  until further analysis. For the investigation of procoagulant EVs in study 2 and 3, two different types of standard pooled plasma (SPP) was created from the blood of one healthy donor. SPP was collected similar to the procedure described above, but the second SPP batch was collected in 4.5 ml specialized tubes containing corn trypsin inhibitor (CTI) tubes (Haematologic Technologies, Essex Junction, VT, USA) with a final CTI concentration of 50  $\mu\text{g/ml}$  once the tube is filled with blood. This is referred to as SPP+CTI.

## **Routine blood tests**

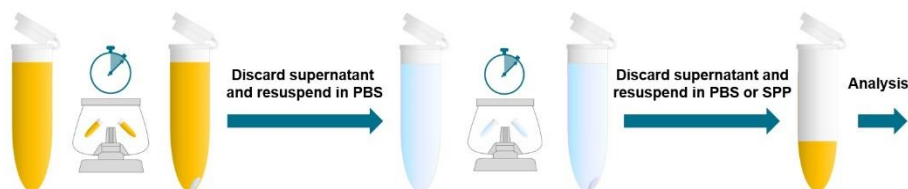
For samples collected from patients in both study 1 and 3 routine blood analyses were included. These analyses comprises standard hemoglobin, erythrocyte, leukocyte and platelet count on a Sysmex XN 9000 system (Sysmex Corporation, Kobe, Japan). Albumin, C-reactive protein (CRP), calcium, and total protein concentrations were measured using the Cobas 8000 C702 module (Roche Diagnostics, Basel, Schweiz). Furthermore, organ specific markers like carbamide, creatinine, pt-estimated glomerular filtration rate, and alanine-aminotransferase (ALAT) levels were measured on the Cobas 8000 C702 module (Roche Diagnostics) to indicate signs of end-organ damage. Coagulation-related analyses include international normalized ratio (INR), activated partial thromboplastin time (APTT), factor FVIII, protein C, fibrinogen, D-dimer, and antithrombin, which all were measured using the ACL TOP 500 CTS (Instrumentation Laboratory Company, Bedford, MA, USA). Measurement

of serum M-protein was performed by gel electrophoresis on a Hydrasys Electrophoresis Unit (Sebia, Lisses, France) or capillary electrophoresis using the Capillaris 2 Flex Piercing system (Sebia) in order to detect M-protein phenotype and any fluctuations during and after the different treatment steps in the designated regimens. Concentrations of free light chain levels were determined by turbidimetry using a SpaPlus analyzer (AH Diagnostics, Tilst, Denmark).

## Isolation of extracellular vesicles

### Study 2

The purpose of study 2 was to set up and validate an EV isolation model, using PFP from healthy volunteers, before moving on to testing the patient samples. EVs were isolated in two steps using DUC on an Avanti J-30i ultracentrifuge with a JA-30.50 rotor and k-factor 280 (Beckman Coulter, Brea, CA, USA). 1 ml PFP was centrifuged at  $20,000 \times g$  (20K) for 30 minutes at  $4^{\circ}\text{C}$ , the supernatant was removed and the pellet resuspended in 200  $\mu\text{l}$  SPP. The supernatant was then centrifuged at  $100,000 \times g$  (100K) for one hour at  $4^{\circ}\text{C}$ , after which the new supernatant was removed and the pellet resuspended in 200  $\mu\text{l}$  SPP. These pelleted EV suspensions are referred to as unwashed pellets, while another batch of samples were subjected to an additional washing step in phosphate-buffered saline (PBS), see Figure 5. In this setting, both 20K and 100K pellets were resuspended in 1 ml PBS after removal of the supernatant and then re-centrifuged at the same settings depending on the pellet type. A batch of pellets were resuspended in 200  $\mu\text{l}$  SPP+CTI for following analysis of the influence intrinsic coagulation.



**Figure 5. The procedure for EV isolation through differential ultracentrifugation.** Plasma was centrifuged to pellet EVs in the bottom of the spin tubes. The supernatant was gently removed from the tube (at a slight angle to avoid pipetting away the pellet material) and the pellet was resuspended in an equal amount of phosphate-buffered saline (PBS). The pellet, now dissolved in PBS, was re-centrifuged at the same settings and the supernatant removed in the same way. Finally, the pellet was resuspended in either standard pool plasma (SPP) or PBS depending on the downstream analysis.

### Study 3

EVs were isolated in the same manner as described for study 2, except all samples were subjected to a washing step prior to the resuspension in SPP.

## Characterization of extracellular vesicles

### Nanoparticle tracking analysis

NTA was used to determine the size and concentration distribution of particles, in the isolated EV pellets in both study 2 and 3. The machinery used was the LM10-HS system with a 405 nm laser (Malvern Instruments, Malvern, UK) attached to a Luca-DL EMCCD camera (Andor Technology, Belfast, UK). The software used was the 3.0 version of the Nanosight NTA software (Malvern Instruments). To determine the optimal capture settings, 0.1  $\mu\text{m}$  Silica Microspheres (Polysciences, Hirschberg, Germany) were applied. The capture settings used in both study 2 and 3 were camera level 10, detection threshold 2, and blur  $9 \times 9$ . A total of five videos with a duration of 30 seconds each was recorded and analyzed for every single sample.

### Western blotting

In study 2 and 3, WB was used to detect specific EV, cell, and lipoprotein markers in the EV suspensions. Proteins were separated using a MiniProtean Electrophoresis System and MiniProtean TGX<sup>TM</sup> 4-15% gels (Bio-Rad Laboratories, Hercules, CA, USA). Prior to separation, the EV suspensions were lysed with 2x Laemmli Sample Buffer (Bio-Rad Laboratories) and boiled for 5 minutes at 95°C. The separated proteins were transferred to Amersham Hybond P 0.20 PVDF blotting membranes (GE Healthcare, Little Chalfont, UK) and blocked with a 5% (w/v) skim milk blocking buffer for one hour. To detect EVs, a primary monoclonal mouse antibody against CD9 (clone M-L13, BD Pharmingen, San Diego, CA, USA) was used in a 1:1000 dilution in blocking buffer. Monoclonal human antibodies against CD38 (daratumumab; Janssen-Cilag A/S, Birkeroed, Denmark) were used for detection of plasma cell marker, i.e. also the MM cells. In order to verify co-isolation of lipoproteins, a monoclonal mouse antibody against apolipoprotein B (clone F2C9, Thermo Scientific, Waltham, MA, USA) were applied. All primary antibodies were diluted 1:1000 in blocking buffer. Secondary antibodies against the corresponding primary antibodies were all horseradish peroxidase conjugated and detected using a Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, IL, USA) and a PXi 4 system and GeneSys software version 1.5.4.0 (Syngene, Cambridge, UK).

### Protein quantitation

In order to address the issue of possible co-isolation and contamination with plasma proteins in study 2, the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) was used and read at wavelength 562 nm Fluostar Optima (BMG Labtech, Ortenberg, Germany). Additionally, a modified version of the activated partial thromboplastin time test (HemosIL and SynthASil, Instrumentation Laboratory,

München, Germany) was run on an ACL TOP 500 CTS system (Instrumentation Laboratory) to detect any co-isolation of FVIII.

### **Transmission electron microscopy and immunogold labelling**

TEM was performed in study 2 and 3 to visualize vesicular structures and detect EV subpopulations through immunogold labeling with antibodies against CD9 and CD38. The method in use is similar to previous studies<sup>243,244</sup>. Samples were loaded on carboncoated and glow discharged 400 mesh nickel grids (SPI supplies, Chester, PA, USA) for 30 seconds. They were then stained in one drop of 1% (w/v) phosphotungstic acid (Ted Pella, Caspilor AB, Lidingö, Sweden) at pH 7.0 and left to dry blotting on filter paper. Visualization of vesicle-like structures was performed on a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) operated at 60 keV. Images were captured with an electron-sensitive CCD camera (KeenView, Olympus, Tokyo, Japan). The use of a grid-size replica (2,160 lines/mm) and the ImageJ 1.50r software (NIH, Bethesda, MD, USA) achieved size-determination of the vesicles. Prior to labelling of EVs, the samples attached to the nickel grids were washed three times in PBS and subsequently blocked with 0.5% ovalbumin (Sigma-Aldrich, St. Louis, MO, USA) in PBS. Primary antibodies against CD9 (clone M-L13, BD Biosciences) and CD38 (daratumumab; Janssen-Cilag A/S) diluted 1:50 in 0.5% ovalbumin-PBS for 30 minutes at 37°C. Then, the samples were washed again and incubated with 10 nm gold-conjugated goat anti-mouse secondary antibodies (British BioCell, Cardiff, UK) in a 1:25 dilution in 0.5% ovalbumin-PBS. After a final washing step, the samples were incubated with 1% cold fish gelatin (Sigma-Aldrich) for 30 minutes, stained with phototungstic acid as described above prior and finally visualized.

## **Coagulation analyses**

### **Calibrated automated thrombogram**

The TG assay used was the well-known and universal test, CAT, that has been described previously by Hemker et al<sup>245</sup>. In this test 80 µl sample were mixed 20 µl prewarmed trigger reagent containing TF and/or PPL. Coagulation was initiated at zero time by adding 20 µl FluCa buffer containing Ca<sup>2+</sup> and fluorogenic substrate (FluCa kit, Thrombinoscope B.V., Maastricht, the Netherlands). The reaction was measured with an automated Fluoroscant Ascent (Thermo Scientific, Waltham, MA, USA). Thrombin Calibrator (Thrombinoscope B.V.) containing a fixed concentration of thrombin-α2-macroglobulin complex, was mixed with the samples, read, and used to calibrate each sample.

## **Study 1**

In study 1, PFP were analyzed for TG using the PPP-low trigger reagent (Thrombinoscope B.V.) containing 1 pM TF and 4  $\mu$ M PPL.

## **Study 2**

In study 2, four different trigger setups were used, in order to identify one or more suitable for investigating procoagulant EVs. Three commercial trigger reagents were used: PPP-low, containing 1 pM TF and 4  $\mu$ M PPL; MP containing 4  $\mu$ M PPL; PRP containing only 1 pM TF (Thrombinoscope B.V.); and the last setup was without addition of any trigger reagent. The samples analyzed for TG were the EV pellets resuspended in SPP or SPP+CTI.

## **Study 3**

In study 3, EV pellets resuspended in SPP were analyzed for TG using the PRP trigger reagent (Thrombinoscope B.V.), containing only 1 pM TF.

### **Circulating cell-free deoxyribonucleic acid assay**

The quantitation of cf-DNA was measured in PFP in study 1, as a surrogate measure for NETs, previously described by Lee et al<sup>246</sup>. DNA was labeled by mixing 100  $\mu$ l PFP 1:10 with PBS and then add 100  $\mu$ l fluorogenic substrate containing 4  $\mu$ M SYTOX Green Nucleic Acid Stain (Thermo Fisher Scientific) followed by 5 minutes of incubation at 27°C. After incubation, the reaction was read with a 485/520 filter set in a FluoStar Optima (BMG Labtech, Ortenberg, Germany) with the Optima software version 2.20R2. UltraPure Calf Thymus DNA (Invitrogen, Carlsbad, CA, USA) was used to generate a standard curve for cf-DNA quantification and normal PBS was used to remove background noise.

### **Procoagulant phospholipid activity assay**

The STA-Procoag PPL kit (Stago, Asnières, France) is FXa-based clotting assay that were used to asses PPL-related coagulation activity. 25  $\mu$ l sample, diluted 1:1 in Owren-koller buffer (Stago), is mixed with PPL-free plasma (Stago). Coagulation was initiated with 100  $\mu$ l trigger reagent containing 0.001 IU FXa and 0.025 M CaCl<sub>2</sub>. The reaction was measured on an automated STA-Compact (Stago) in accordance with the protocol from the manufacturer.

## **Study 1**

In study 1, PPL activity was measured in PFP.



## Study 2 and 3

In study 2 and 3, EV pellets resuspended in SPP were analyzed for PPL activity.

### Microvesicle tissue factor assay

The modified MV-TF assay used in study 2 and 3 were based on a method described by Wang et al<sup>238</sup>. Before measurement of MV-associated TF activity commenced, 600  $\mu$ l plasma was diluted in HBSA buffer containing 137 mM NaCl, 5.38 mM KCl, 5.55 mM glucose, 10 mM HEPES, and 0.1% (w/v) bovine serum albumin. The mixture was then centrifuged at  $20,000 \times g$  for 15 minutes at 4°C to spin down MVs. After removing the supernatant, the pellets were washed once in HBSA, whereafter the pellets were resuspended in 180  $\mu$ l HBSA. The MV pellet suspensions were incubated with monoclonal mouse anti-CD142 antibody (clone HTF-1, BD Pharmingen) or IgG from mouse serum (Sigma-Aldrich) for 15 minutes. To trigger coagulation, 50  $\mu$ L HBSA containing 10 mM CaCl<sub>2</sub>, 73 nM FX (Enzyme Research Laboratories, South Bend, IN, USA) and 2.4 nM factor VIIa (Enzyme Research Laboratories) was added and the samples were left to incubate at 37°C. After two hours of incubation the coagulation was stopped by adding a HBSA stop solution, containing 25 mM EDTA and 25  $\mu$ l buffer, containing 4 mM Pefachrome FXa 8595 (Pentapharm, Basel, Switzerland) was added for another 15 minutes of incubation at 37°C. The reaction was detected using a Fluostar Optima (BMG Labtech) at absorbance 405 nm. To finally calculate the MV-TF activity, a standard curve was created using Innovin (Siemens Healthcare, Erlangen, Germany).

### Statistics

In all three studies, Mann-Whitney U test or Student's t-test was used to find significance dependent on the type of distribution. Correlations analysis between different analytical outcomes were performed using the Pearson correlation coefficient. For the comparison of differences during the different treatment regimens in study 1, Wilcoxon matched-pairs signed rank test was applied. In study 2, differences between washed and unwashed pellets were identified using the paired samples t-test. All statistical analysis throughout the three studies were performed using the IMB SPSS Statistics 23 software (SPSS, Chicago, IL, USA) and/or Graph Pad Prism 6 and 7 (GraphPad Software, La Jolla, CA, USA).

## Chapter 6. Results

### Study 1

#### *Prothrombotic abnormalities in patients with multiple myeloma and monoclonal gammopathy of undetermined significance*

Thøger Nielsen, Søren Risom Kristensen, Henrik Gregersen, Elena Manuela Teodorescu, Jonas Ellegaard Nielsen and Shona Pedersen

#### **Hypercoagulability in MGUS and MM**

##### **Thrombin generation**

Significantly increased TG was detected in both MGUS and MM, depicted by an increase in ETP (approximately doubled) and peak height (approximately four times higher) when compared to control subjects. Lagtime and time-to-peak were significantly reduced to almost half the time in both MGUS and MM.

##### **Procoagulant phospholipid activity**

The PPL activity in were increased in MGUS and MM, however, with the most significant increase in the latter. The PPL clotting time was by 46% for the MGUS patients and by 61% for the MM patients.

##### **Microvesicle-associated procoagulant activity**

The only significant TF activity associated to MVs were detected in the MM patients, which presented with higher heterogeneity compared to the MGUS patients and control group. The MGUS showed an insignificant trend, where some MGUS patients had increased levels of TF activity in comparison to the control group. Almost none of the control subjects had any detectable level of TF activity. No correlation between MV-associated TF activity and TG was detected.

##### **Cell-free deoxyribonucleic acid**

The levels of cf-DNA in plasma were only found to be significantly increased in MM patients with a median increase of 73 ng/ml (42%). This increase may result from the heterogeneity that the MM patients hold, where some individual patients reached levels 10 times higher than the lowest in the group.

## **Effect of treatment on the global coagulation in MM**

Overall, we did not find any apparent effects of the conventional treatment or HDCT with ASCT on thrombin generation. When a comparison between the few patients that finished a whole sample collection schedule were made, some minor differences were detected. Lagtime and time-to-peak were slightly increased after VCD induction in the HDCT+ASCT group. For the conventional treatment group, peak height increased slightly after 6 months and ETP increased slightly after 12 months.

## **Study 2**

### *Investigation of procoagulant activity in extracellular vesicles isolated by differential ultracentrifugation*

Thøger Nielsen, Anne Flou Kristensen, Shona Pedersen, Gunna Christiansen and Søren Risom Kristensen

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## **Isolation and characterization of extracellular vesicles**

### **Size and quantity of isolated particles**

After applying the isolation procedure on plasma samples from control subjects, the concentration of particles measured with NTA in the pellet suspensions were reduced to between 5-8% of that in the original plasma. The mean particle size increased by 40% from plasma (85 nm) to the 20K pellets (119 nm) and by 25% to the 100K pellets (109 nm). The fraction of particles larger than 100 nm in size was close to 50% in both 20K and 100K pellets. There were slightly more particles above 200 nm in size in the 20K pellets. TEM images confirmed the presence of several EV-like structures. Many smaller vesicle-like structures were also identified in the unwashed pellets, many of which resided in proximity of the larger ones.

### **Surface proteins**

Several CD9-positive EV-like structures were identified in both 20K and 100K pellets through immunogold-labeling. WB further confirmed this though detection of CD9 in both pellet types.

### **Protein contaminants**

WB revealed the presence of apolipoprotein B in the isolated pellets, especially the 20K pellets. The overall protein content in the pellets was measured to be between 1200-2200 µg/ml.

### **Effect of washing the pellets**

After washing of the pellets with a PBS washing step, the particle levels decreased by 37-38% in the both 20K and 100K pellets. However, the mean size increased by 33% in the 20K pellets and by 19% in the 100K pellets. Moreover, the fraction of particles below 100 nm in size shrink from more than 50% on average in all pellets, to less than 10% in the 20K pellets and ~30% in the 100K pellets. Apolipoprotein B content was not detectable by WB after washing and the protein content reduced by more than 88%. No co-isolation of FVIII was detected in 20K or 100K pellets. We found little to no effect on TGA results between washed and unwashed pellets resuspended in SPP.

### **Procoagulant activity exerted by extracellular vesicles**

#### **Effect of extracellular vesicles on thrombin generation**

Overall, an increased TG was observed in SPP spiked with with EVs from 20K and 100K pellets, however, the results varied for each type of different trigger stimuli used. When using no trigger reagent or the MP reagent (4  $\mu$ M PPL), TG seemed to increase independent on pellet type used, but the effect was difficult to evaluate due to high inter-individual variation. Using PRP (1 pM TF) as trigger reagent, TG is noticeably increased in both pellet types, but with the greatest effect in the 20K pellets. When using PPP-low (1 pM TF and 4  $\mu$ M PPL) as trigger reagent, the procoagulant effect of the 20K pellets disappear in contrast to the 100K pellets that remained elevated.

Analysis of TG in pellets resuspended in SPP containing CTI showed that no TG occurred using no trigger reagent or the MP trigger reagent. The addition of CTI seemed not to influence TG measured with PRP or PPP-low, except for eliminating the procoagulant effect of the 100K pellets observed in the PPP-low setup.

When measuring TG in the original plasma, from which the pellets were isolated, and the corresponding supernatants after the DUC isolation procedure, a significant reduction in TG was observed.

#### **Effect of extracellular vesicles on procoagulant phospholipid activity**

Analysis of PPL activity demonstrated a significant effect of the pellets. 20K pellets reduced the PPL clotting time by almost 50%, whereas the 100K pellets reduced the PPL clotting time by almost 20%. The PPL activity correlated with the TG measured with the PRP trigger reagent, but this effect disappeared when the trigger was replaced with PPP-low trigger reagent (also containing PPL). Furthermore, the PPL activity correlated to the mean particle size, with the highest PPL activity in pellets with the largest mean particle size. Similar to the analysis of TG, PPL activity was reduced in the supernatants after pellet isolation from the original plasma of the control subjects.

## Study 3

### *Extracellular vesicle-associated procoagulant phospholipid and tissue factor activity in multiple myeloma*

Thøger Nielsen, Søren Risom Kristensen, Henrik Gregersen, Elena Manuela Teodorescu, Gunna Christiansen and Shona Pedersen

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### **Isolation and characterization of extracellular vesicles**

#### **Nanoparticle tracking analysis**

Overall, significantly more particles were found in the pellets from MM patients compared to the control group, where the 20K pellets contained the most particles. In both the control group and the patient group, the 20K pellets had the highest mean particle size, although the difference between the two pellet types was most pronounced for the MM patients. In general, the 20K pellet from the MM patients had the highest fraction of particles above 100 nm and above 200 nm in size.

#### **Transmission electron microscopy, immunogold-labeling and western blotting**

TEM images revealed EV-like structures in all pellet types from both patients and control subjects. Moreover, CD9-positive EVs were detected in both 20K and 100K pellets from control subjects and MM patients, but most distinct in the 20K pellets of the MM patients. A rough estimation revealed that the CD9 content was 3-6 fold increased in MM pellets when compared to those of the control subjects. CD38-positive EV populations detected in both control and MM pellets, but it was most pronounced in those from MM patients.

### **Procoagulant extracellular vesicle activity in multiple myeloma**

#### **Thrombin generation**

The measured effect of EVs on TG was found only to be present in the 20K pellets from the MM patients. Here, peak height was more than doubled and ETP increased by almost 50% compared to the control pellets. Furthermore, both lagtime and time-to-peak decreased by approximately 30%.

#### **Procoagulant phospholipid activity assay**

The PPL activity was increased only in the 20K pellets of the MM patients, where the reduction in PPL clotting time was nearly 50%.

### **Microvesicle-tissue factor assay**

In line with study 1, most control subjects showed no detectable MV-TF activity. Most of the MM patients, however, showed increased MV-TF activity with some individuals depicting a marked increase compared to the rest.

### **Effect of treatment on procoagulant extracellular vesicle activity**

After receiving anti-myeloma treatment the procoagulant activity of EVs in the 20K pellets of MM decreased. It seemed that patients receiving VCD induction therapy, prior to their HDCT with ASCT, had a more distinct response compared to the MM patients receiving conventional therapy. This was, for the conventional group, depicted in a lowered almost 30% lower ETP and peak height and almost no reduction in lagtime and time-to-peak. In contrast, the VCD group had a 56% lower ETP, 42% lower peak height, and an approximately 30% increased lagtime and time-to-peak. Furthermore, the VCD group had a significant and almost complete reduction in PPL-clotting time compared to a modest response in the conventional group. Both treatment groups also showed an insignificant trend of reduction in their particle concentration post-treatment, especially in the VCD group having their fraction of particles above 200 nm in size almost cut in half.

## Chapter 7. Discussion

The aim of this thesis was to investigate the hypercoagulability present in patients with MM and MGUS and any possible association to an increased VTE-risk. We aimed to investigate several procoagulant factors potentially responsible for this hypercoagulability at diagnosis and throughout the treatment period. Furthermore, we aspired to optimize and validate a model that would allow for a proficient investigation and concise exploration into the role of procoagulant EVs. Eventually, we would use the model to explore any procoagulant activity of EVs in the MM patients.

We found that patients with MGUS and MM displayed hypercoagulability, consisting of increased TG and PPL activity. MM patients had increased levels of cf-DNA, as an indication of NETs. Treatment of multiple myeloma did not seem to have a significant impact on TG in any of the treatment arms and NETs levels seemed to drop to normal levels. We optimized a model for effective investigation of procoagulant EVs from plasma and found that procoagulant EV activity were present in MM at diagnosis, but that this activity decreased after the first treatment with most effect in patients receiving VCD induction therapy prior to HDCT and ASCT.

The topic of the first part of the discussion revolves around the methods of choice. It will entail an assessment of the methods chosen to test for coagulation abnormalities in the different studies. It further elaborates the main issues and thought processes behind the systematic optimization of a model for isolation and functional analysis of procoagulant EVs performed in study 2 and 3. The second part of the discussion entails the investigation and comparison of hypercoagulability in plasma from MGUS and MM, and healthy controls (study 1). This comprises the role of NETs, TF and PS, particularly in relation to procoagulant EVs (study 3). The discussion of hypercoagulability in patients with MM will entail the assessment of different anti-myeloma treatments and their potential role in hypercoagulability and possible VTE-risk.

### Methods of choice

#### Thrombin generation

The choice to use a TG assay in the analysis of hypercoagulability was based on its capability to depict a global evaluation of the hemostatic system, including information about the initiation of coagulation, amplification and propagation, and resolution phases. We measured TG with CAT, because it is proficient in demonstrating changes in most coagulation factors and the effect of anti-coagulant treatments and it is an acceptably standardized method<sup>245,247</sup>. It has been used

frequently to detect hypercoagulability in several instances, including investigation of acute deep vein thrombosis and thromboembolism<sup>248–250</sup>. Several commercial reagent kits are available with different concentrations of TF and PPL that theoretically can be used to demonstrate different changes of PPL and TF activity in plasma samples. For all samples collected in the MEXO study, the centrifugation protocol used was recommended by Lacroix et al.<sup>224</sup> and the product is considered to be PFP. Since there is no kit available for PFP, our choice was to use the PPP-low reagent with 1 pM TF and 4  $\mu$ M PPL to analyze our plasma samples. The CAT method has previously been used to investigate procoagulant properties of EVs, and a commercially available reagent exists, the MP kit (short for microparticle) containing only 4  $\mu$ M PPL. Gheldof et al.<sup>235</sup> used CAT with the MP kit to demonstrate procoagulant activity of MVs from a cultured breast cancer cell line. In study 2, where we investigated procoagulant effect of EVs in healthy subjects, the concentrations TF and PPL used to trigger the reaction was definitely of importance and not simply chosen. We were, however, not able to achieve any reliable data on MV-associated procoagulant activity, when we used no trigger reagent or the MP reagent in these healthy volunteers, because of high inconsistency and absence of TG when tested with addition of CTI. Luddington et al.<sup>251</sup> show that addition of CTI can be used for investigation of low concentrations of external triggering TF, which nevertheless is consistent with our findings. We observe no TG in plasma with CTI, until we use a reagent containing 1 pM TF or more. Furthermore, we learned that adding too much external PPL via the trigger reagent would conceal or diminish a big part of the procoagulant effect of EVs (presumably a majority of the PPL activity). These observations were the basis for the decision to use PRP kit with only 1 pM TF for investigation of procoagulant activity of EVs resuspended in SPP.

### Procoagulant phospholipid assay

The procoagulant phospholipids, essential for binding of the tenase and prothrombinase complexes, is mainly residing on activated platelets and MVs. When we tested PFP with a procoagulant phospholipid assay, we expected the source for PPL activity to be almost purely MV-associated and without any interference from activated platelets. A commonly used method to detect PS-positive MVs is FC with Annexin V that binds PS, but this method measures the number of PS-positive MVs (not activity) and may furthermore have limitations in detecting the smallest of MVs that also may bud from the cell membrane<sup>252,253</sup>. Several alternative PPL activity assays are also frequently used to assess the MV-associated PPL activity in plasma<sup>253</sup>. We used the clot-based STA-Procoag-PPL kit due to its simplicity and reproducibility and it has been shown that it correlates well with FC with Annexin V<sup>254</sup>. The PPL activity assay depicts the total plasma PPL activity and cannot distinguish whether large and/or small EVs. We included the assay in study 2 and 3 to measure the total PPL activity, but also to check for correlation other EV-related measures, e.g. mean particle size or concentration.



### **Cell-free DNA marker for neutrophil extracellular traps**

Since NETs is an emerging factor of interest in regards to thrombosis and is capable of trapping MVs carrying PS and/or TF, we included the quantification of cf-DNA as a surrogate measure for NETs levels. Previously, microscopic techniques using antibodies against constituents of NETs were the typical method of choice for investigating NETs, but most studies using this approach required a preceding isolation of the neutrophils<sup>255</sup>. Real-time PCR-, ELISA-, and FC-based methods can be applied to investigate cf-DNA or NETs components, like myeloperoxidase or neutrophil elastase (NE), in body fluids<sup>256–258</sup>. Lately, the fluorescent nucleic acid stains for cf-DNA has more frequently been used as a surrogate measure of NETs in serum and plasma<sup>259,260</sup>. The method is time-efficient and relatively easily standardized, but should be interpreted with caution, because it measures cf-DNA without defining the source, i.e. the cause of cell death or cell of origin<sup>255</sup>. We find the method relatively stable with a control population showing homogenous levels of cf-DNA that for the most part was around 200 ng/ml. The literature has been reporting levels of approximately 10-200 ng/ml dependent on preanalytical preparations<sup>246,261</sup>. How much of the quantitated cf-DNA that in fact is associated to NETs is unclear and should therefore be evaluated with caution.

### **Microvesicle-associated tissue factor activity assay**

An MV-TF activity assay was included in both study 1 and 3 since it was designed for measurement of MV-associated TF activity. Two MV-TF activity assays exists (kinetic and end-point), but they seem to have issues regarding sensitivity when measuring low levels of TF (e.g. comparing patients to healthy controls)<sup>262,263</sup>. In addition, it has also been documented that the MV-TF activity in healthy subjects are below the limit of detection for this assay, thus rendering a comparison between healthy and pathological samples challenging<sup>264,265</sup>. We adapted an MV-TF activity assay from the Chapel Hill group<sup>238</sup>. This assay did prove issues in detecting TF activity in many of the samples, especially in the control group. Therefore, the results of this analysis was difficult to compare to our other analyses and serve exclusively be used to identify individual patients with high MV-TF activity. A shortcoming of the method is the lack of sensitivity, but recently a study was published describing how to improve the sensitivity of the MV-TF method without loss of specificity<sup>266</sup>. We chose, in parallel to the MV-TF assay, to isolate EVs from plasma from both patients and controls for procoagulant analysis of EVs with other coagulation tests and characterization tools.

### **Isolation of extracellular vesicles**

Since there are many opinions on how to isolate EVs most efficiently, it can be difficult to find a model that fits all downstream analysis. Initially, we applied the PFP extraction procedure recommended by Lacroix et al<sup>224</sup>, which was performed at room

temperature to avoid platelet activation (and thereby additional EV secretion), and to ensure a platelet-free medium for EV isolation. We then chose to investigate the strengths and limitations of a DUC-based EV isolation technique, since it theoretically enables for isolation of more distinct MV and exosome subpopulations. Furthermore, it would enable us to resuspend the isolated EVs directly in a set amount of SPP, which we needed for our downstream coagulation analyses. The requirement for resuspension of the isolated EVs in SPP impeded any thoughts of including a SEC separation. That way, the EVs would presumably be diluted to an extent where we would be unable to measure their procoagulant properties with our selected panel of coagulation assays. Filtration was excluded based on the putative loss of material (e.g. clotting of the filter) and potential risk that the procedure may disrupt the integrity of the EVs and distort their functional abilities. Instead of including an additional isolation technique, we added a washing step to the DUC procedure. As recommended by Thery et al.<sup>267</sup>, washing of the pellets achieved through DUC in equal volumes of PBS is an almost unavoidable step to include, due to the complex nature of plasma. The washing of the pellet should potentially remove many smaller contaminants, like protein aggregates and lipoproteins. It is well known that many lipoproteins overlap in size and density with EVs and are known as a major constituent of the total amount of particles measurable in plasma samples<sup>227</sup>. Therefore, we also included several characterization tools to investigate the extent of coisolation of protein aggregates and lipoproteins.

### **Characterization of extracellular vesicles**

While NTA frequently is used to quantitate EVs in different mediums, we included it well aware that it may serve as a rough estimate EV quantity and size due to its sensitivity towards contaminating factors. We assumed that washing of the pellet would increase the validity of the particle concentrations and sizes measured in the 20K and 100K pellets. NTA revealed an overlap between the two pellet types, but did also show an increase in median particle size, especially after the washing step. The considerable particle size overlap of particles between 100-200 nm in size is similar to findings by Kowal et al.<sup>193</sup> made on cell culture supernatants. After washing of the quantity of particles in the pellets decreased by approximately 95% and most of the particles removed are from the fraction <100 nm in size. This indicates, that we probably were removing many lipoproteins and maybe small exosomes that we would not expect would contribute significantly to procoagulant EV activity. We determined that the particle levels measured using NTA would not be suitable to standardize the amount of EVs resuspended in SPP from each patient or control, because surely the NTA measurements was still influenced by some contaminants. WB was mainly introduced in the studies to confirm the presence of CD9- and CD38-positive EV populations and verify the removal of some apolipoprotein. WB is a tool frequently used to detect, e.g. tetraspanin-enriched EVs and, it is suited for spotting relevant contaminants<sup>193</sup>. However, without a valid standardization of the amount of EV-material loaded onto the gel, the differences between samples should only be used to

make a rough relative estimation with caution. To support the findings of WB, TEM and immunoelectron labeling were applied as a 'seeing is believing' tool, in a similar manners as others<sup>235,268,269</sup>. TEM and immunogold labeling of EVs was not used to estimate the amount of CD9- and CD38-positive EVs, but both TEM and WB was also used to confirm the effect of washing on the contaminating protein aggregates and lipoproteins. In general, the optimization of an EV isolation model partly achieved some size and phenotypical separation of EV populations with reduced amounts of contaminants, and the EVs showed clear signs of procoagulant activity.

## **Hypercoagulability in MGUS and MM**

Our findings in study 1 supported our hypothesis that patients with MM and MGUS have increased procoagulant activity in the blood compared to healthy subjects. We find that TG, measured in plasma drawn from MGUS and MM patients at diagnosis, was markedly increased compared to healthy subjects. In addition, we find that both patient groups have markedly increased PPL activity, however, only MM patients show tendencies of increased MV-TF activity and increased levels of NETs. Throughout anti-myeloma treatment, we find little or no effect of the treatment on the hypercoagulable state observed at diagnosis. Our data indicated that elevated TF and PPL activity, especially associated to EVs, may play a role in the MM-related hypercoagulability, but this effect is lowered after the first cycle of treatment, thus supporting our second hypothesis that MM in comparison to healthy subjects have elevated blood levels of procoagulant EVs.

## **Procoagulant factors in MGUS and MM**

The elevation of TG that we demonstrated in MM and MGUS at diagnosis, indicate that parts of the global coagulation system is out of balance and in turn may be a contributor to the reported VTE-risk associated to both conditions<sup>55,56,137</sup>. Few studies have described TG in MM or MGUS. Two studies described little to no difference in TG in plasma from MM patients when compared to healthy controls<sup>270,271</sup>, whereas another study reported an unexpected attenuation of TG in MM compared to healthy subjects<sup>150</sup>. One study finds ETP and peak thrombin increased in both MGUS and MM, with the largest increase in the latter<sup>272</sup>. An issue to address regarding the lack of consensus may be found in the pre-analytical and analytical choices made. Firstly, all studies use platelet-poor-plasma, which presumably do contain more residual platelets in comparison to PFP. Secondly, two studies use the PPP reagent, which contains 5 pM TF instead of 1 pM. Together, these factors may hide a potential increase in TG like the one we observed in our study 1. One study finding an increase in TG uses the same TGA setup as we do, using the PPP-low reagent, suggesting that lower levels of triggering TF increases the assay sensitivity to differences between healthy subjects and patients. MGUS patients presented with increased TG, although not as prominent as the MM patients, which is in line with the findings of Crowley et al.<sup>272</sup>. The same is the case with plasma PPL activity, which raises the question if

MGUS and MM may possibly share some underlying mechanisms that cause hypercoagulability. Whether this truly is due to shared features in pathophysiology of the underlying diseases or progression mechanisms associated to cancer that affects the coagulation systems may have clinical value. MGUS, being asymptomatic, is a condition typically detected through hospital visits regarding other conditions. Therefore, some may have some other underlying conditions that may influence our results. Nevertheless, these are speculations and not questions we can answer in this thesis.

NETs are known to be expressed more in some tumors, like lung cancer, but otherwise the knowledge of NETs in cancer development is sparse. Recently, it was proposed that the tumor environment induces NETs release from neutrophils, where they may play a role in adherence and activation of platelets<sup>273</sup>. In regards to MM, almost no knowledge has been published on the subject. A study reported low levels of citrullinated histone H3, another surrogate measure for NETs, in MM suggesting that NETs play a minor role in MM-related VTE-risk<sup>274</sup>. Our analysis of cf-DNA showed that approximately one third of the MM patients had elevated levels at diagnosis. This could indicate that NETs do have a role in MM-related hypercoagulability for some individual patients, but the absence of VTE hinders any further conclusion. Some of the cf-DNA measured may also be footprints from excess cellular decay or apoptosis related to MM or comorbidities.

### **Extracellular vesicle mediated procoagulant activity in MM**

In study 1, increased plasma PPL activity in most MM patients indicates presence of procoagulant PS-positive MVs. No correlation was evident between PPL activity and TG, which could indicate that other blood coagulation factors are also contributing to the increased TG. Furthermore, the increased MV-TF activity also indicate the presence of procoagulant TF-positive EVs in many of the patients. This did not correlate to TG, but the effect of the MV-related TF activity may likely have been hidden by the TF from the trigger reagent.

The results on EV ‘spiking’ in SPP indicated that MM is associated with procoagulant EVs, likely in the shape of MVs containing TF and PS. A subpopulation of CD9-positive EVs were most abundant in the procoagulant fraction, i.e. 20K, suggesting that the majority of EVs, including the larger procoagulant MVs, possibly are isolated in this fraction, whereas the remainder, probably the smallest and non-procoagulant EVs and contaminating factors may constitute a considerable part of the 100K fraction. To our knowledge, the impact of procoagulant EVs in MM is an almost unexplored subject. Auwerda et al.<sup>149</sup> has previously demonstrated an increased MV-associated TF activity in MM using a FXa-based method on EVs isolated in a similar manner as our 20K pellets. Due to both different preanalytical procedures and EV-isolation deviations, a direct comparison of the results is complicated. Fotiou et al.<sup>275</sup> reported on an overall increased PPL-clotting time in plasma of MM patients similar

to our findings on the subject, but do not include any profound analysis of the impact of procoagulant EVs in their paper.

Specific procoagulant phenotypes of cancer cells showing over-expression of TF and PS has also been described<sup>276,277</sup>. The procoagulant cancer cells are also known to secrete EVs containing TF and PS<sup>278–280</sup>. Several studies report that procoagulant EV-activity exists in various types of cancers, underlining that the procoagulant EVs are an inevitable factor to consider in cancer-related hypercoagulability and VTE risk<sup>281</sup>. As a therapeutic target for MM, CD38 is overexpressed on MM cells and most likely on some EVs secreted from these cells<sup>282</sup>. It is being investigated if CD38 positive EVs released from MM cells is another mechanism to avoid immunodetection among other cancer features<sup>283</sup>, but no reported connection between CD38-positive EVs and EV-related procoagulant activity in MM exist. In line with this, we found that most CD38 was present in the isolates containing the majority of small EVs (i.e. 100K pellets), which suggests that the procoagulant activity of EVs we observe is not linked directly to CD38-positive EVs from MM cells. Yet, the procoagulant EV activity may be linked to cancer EVs that do not contain CD38. It is also feasible that other cancer-induced effects (e.g. inflammation and altered protein metabolism) is connected to the EV-mediated hypercoagulability we observe in MM.

None of the patients developed VTE in their period of participation in the MEXO-project, which unfortunately make it impossible to substantiate any connection between procoagulant EVs and MM-related VTE-risk. However, we demonstrate that MM patients do have increased EV-mediated procoagulant activity, but with a considerable variation among patients. Auwerda et al.<sup>149</sup> touches the subject of a possible connection between MV-TF activity and VTE in MM patients, just like procoagulant EVs and the association to the VTE-risk has been described in several other cancer types. This emphasizes the general knowledge that cancer in it self entails a significant risk of VTE possibly caused by many factors, where procoagulant EVs only represents one of its many risk-associated arms.

### **Anti-myeloma treatment and its implications on coagulation**

Our data on coagulation activity during treatment suggest that neither MPV nor HDCT+ASCT treatment have any significant impact on plasma TG. Even though some anti-myeloma regimens are associated with increased VTE risk, this observation is in line with others reporting no effect of treatment on TG in MM<sup>284</sup>. There is, however, some controversy on the topic. Another group showed a minor increase in TG in patients receiving mainly IMiDs and they suggest velocity index as an indicator of hypercoagulability, but neither ETP nor peak thrombin were increased<sup>271</sup>. Recently, Leiba et al. suggested that TG may serve as a potential predictor of VTE by demonstrating increased TG in MM patients with a future thrombotic event compared to those without<sup>270</sup>. In this case, more than 80% of their observed thrombotic events occurred during treatment with IMiDs, which potentially may be part of the cause. In

the past decade, the VTE-associated IMiDs are getting replaced with drugs like bortezomib that is less thrombogenic<sup>285</sup>. This is the case for more than 75% of the patients receiving conventional therapy in our study 1, which may also explain why we detect no treatment-related hypercoagulability.

We further found that individual patients have high levels of cf-DNA at the time of diagnosis, but these almost diminished to the same level as the other patients had after being treated with MVP or HDCT with ASCT. This suggests that there may be a connection between treatment and decreasing levels of NETs, but whether this connection is direct/indirect or related to NETs-associated procoagulant activity remains to be determined. Even though no published data on NETs in MM exist, it is known that cancer cell-induced NETs are connected to cancer progression<sup>286</sup>. Therefore, it may be feasible that the reduction in cf-DNA we observe in MM patients simply is due to a decrease in stimuli from cancer cells that are killed in response to treatment.

In study 3, we demonstrated a distinct procoagulant effect of EVs isolated from plasma from MM patients. This effect did conversely diminish markedly after the initial treatment phase, but most apparent in the group receiving VCD induction therapy. A similar trend was observed by Auwerda et al. in MM patients after treatment<sup>149</sup>. The reduction of procoagulant activity of EVs we observed seems to be related to the larger MVs, but also the cancer. Maybe they originate from MM cells or they are a result of the systemic response to the cancer. It is likely that the reduction of procoagulant activity is connected to the overall state of health of the MM patients that is generally increasing (reflected by treatment response), particularly in patients receiving VCD.

Overall, our data suggest that the anti-myeloma treatment used in our study is not worsening the hypercoagulable state detected in MM patients at diagnosis. Nevertheless, we were not able to identify individual patients with elevated VTE-risk during treatment, which may benefit from thromboprophylaxis, without data from any patients who developed VTE.

## Chapter 8. Conclusions

Overall, the studies suggest rejection of our null hypotheses by indicating that hypercoagulability do exist in patients with MGUS and MM and that this hypercoagulability, to some extent, can be attributed to procoagulant EVs.

In study 1, we found evidence of hypercoagulability in patients with MGUS and MM related to TG and PPL activity, suggesting that some connection between the underlying diseases and the increased procoagulant activity exist. Some MM patients have increased levels of cf-DNA and MV-associated TF activity at diagnosis, which indicates that both NETs and MVs may have a role in the MM-related hypercoagulability. During and after anti-myeloma treatment, TG for the most part remains unchanged. This trend is also applicable for cf-DNA levels except for a few deviating patients that stabilize during and after onset of treatment.

In study 2, we optimized a DUC-based model for isolation of EVs intended for downstream coagulation analysis. We learned that a single washing step would remove a major part of the co-isolated contaminants, e.g. lipoproteins and protein aggregates, without pronounced loss of EV-material. We were able to identify a subpopulation of EVs positive for CD9 and that the isolated EVs were capable of altering coagulation in a procoagulant direction when analyzed with a combination of CAT and PPL activity assay. Our findings also suggested a connection between size of EVs and procoagulant activity, i.e. the larger MVs exerted increased procoagulant activity.

In study 3, we find evidence of CD9- and CD38-positive EV subpopulations. Furthermore, we found a procoagulant population of EVs, most likely MVs, which attributed to hypercoagulability through increased TF and PPL activity in MM patients. The procoagulant EVs possibly have a connection to the cancer cells, directly or indirectly, but presumably not through CD38-positive EVs. The EV-mediated procoagulant activity decreases after onset of anti-myeloma treatment. The effect is particularly evident in MM patients receiving VCD induction therapy, and suggests association to the overall health improvement of the patients in response to the therapy.

## Chapter 9. Perspectives

Since the recognition of EVs as important bearers of various biologically active molecules and their potential roles in numerous diseases, the interest in the field has ever since grown and advanced, especially after the founding of ISEV in 2012 and the establishment of guidelines to advance the understanding and research of EVs. However, EV research is still a young field of research and much attention is directed towards standardization of methodology in regards to EV isolation, characterization and functional analysis<sup>200,263</sup>. Members of both ISEV and The International Society on Thrombosis and Haemostasis have also recognized the need for development and optimization of methods that are suited for investigation of EVs and their role in hemostasis and thrombosis<sup>287,288</sup>.

Issues of method optimization and advancement for EV research has also been a major topic in this thesis. Although, several tools have been developed for EV isolation they all have pros and cons in regards to the purpose of subsequent investigation plans. We utilized a DUC model and initiatives from already existing protocols to create our own version suitable for our methods of choice for coagulation testing. However, it is very likely that we can refine our EV isolation procedure, e.g. in regards to centrifugation duration and before or after SEC/magnetic bead separation. Moreover, we could possibly refine our methods for characterization of EVs to get a deeper understanding of the origin of the EVs, and if and how they relates to the cancer. This may be achieved through reassessment of EV- and disease markers, choice of antibodies, or by including techniques like FC and cryo electron microscopy, that currently also are being developed and optimized for EV investigation.

The investigation of EVs and their role in relation to hypercoagulability and VTE in cancer steadily improving, but procoagulant EVs in MM is a rather undescribed chapter, except for the paper from Auwerda et al.<sup>149</sup>. Unfortunately, can neither they nor we link procoagulant EVs to VT-risk. Just as it applies for studies with limited availability of patients, the lack of VTE events in our studies pose a problem from an investigative point of view. Future investigations on the matter may call for further biobanking of MM samples, a possibly a re-evaluation of inclusion criteria, and a solid analysis plan for efficient analysis with loss of sample quality and comparability. The same considerations do also concern the investigation of treatment effect on hypercoagulability, since MM is a patient group with a high drop-out potential due to the high mean age and variable disease stages.

TF-positive EVs are also becoming interesting target in regards to development of novel medicines<sup>289</sup>. For MM, a potential candidate already exists in daratumumab (anti-CD38 immunotherapy against plasma cells), which are used more often in anti-myeloma treatment due to promising treatment response. To investigate whether the



therapy activates the immune system to target EVs from cancer cells and how it affects the procoagulant activity of EVs may be of clinical value.

We believe that some of these considerations may help advance the investigation of procoagulant EVs in MM, elucidate their possible relationship to VTE-incidence and aid in identifying patients that may benefit from an early thromboprophylactic measures.

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